

Cytoskeletal Determinants of Synaptic Morphology

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Prof. Marcel Tanner

Dedicated to my mother

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GENERAL INTRODUCTION

The central nervous system (CNS) is an assembly of cells that constantly perceive, analyze and integrate information from the outside world. To achieve its task of directing various aspects of behavior as well as controlling organ function, the nervous system transfers signals via ionic currents through nerve cells, or neurons, the functional units of electrical transmission in living organisms. Neurons possess incontestably the most diverse and sophisticated shape of all cells. They are highly polarized and send out a number of processes interconnecting individual cells to form a remarkably complex network. There is extraordinary morphological heterogeneity amongst neurons with shape being directly linked to the function a particular neuron has to perform. However, one feature is common to all nerve cells.

Neurons are made of three distinct domains; the cell body, the dendritic tree and the axon (Figure 1). Dendrites and axons enable neurons to receive or send information respectively and they differ in molecular, morphological as well as functional terms. Axons, the signal forwarding unit, are myelinated and often travel long distances until they reach their target, which they contact via specialized presynaptic terminals containing vesicles filled with neurotransmitters, the chemical messengers of electrical signals. In contrast, dendrites, the information receiving units, do not extend far from the cell body and often branch extensively. Upon proper contact with an axon, postsynaptic specializations form on dendrites to create functional synapses with the presynaptic terminals of axons. On most excitatory synapses in the brain these contacts are made on small dendritic protrusions extending

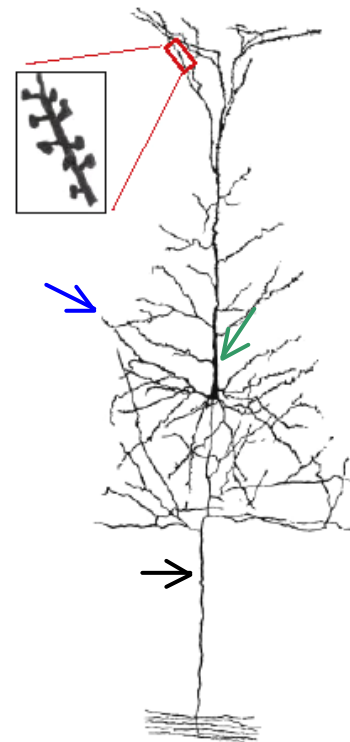


Figure 1: Camera lucida drawing from a pyramidal neuron showing its three distinct domains. Cell body (green arrow), axon (black arrow) and dendritic tree (blue arrow). Dendritic spines are enlarged in the red box.

(Adapted from Ottersen and Helm 2002.)

from the dendritic shaft, termed dendritic spines (Harris, 1999), which are thought to play an important role in learning and memory.

1 Dendritic spines:

First described by Ramon y Cajal, dendritic spines are small, specialized dendritic protrusions which act as integrating units of synaptic input for a diversity of neurons. Dendritic spines, consisting of a bulbous head connected to the dendritic shaft by a narrow neck, are widely distributed in the mammalian brain. Their shapes differs over a wide range of morphologies ranging from short to long, thin to thick or headless to bearing large head structures. On the basis of early ultrastructural analysis, different types of dendritic protrusions have been classified into four major categories according to their morphology: thin, stubby or mushroom-shaped spines and filopodia (Figure 2) (Chang and Greenough, 1984; Chicurel and Harris, 1992; Peters and Kaiserman-Abramof, 1970). Despite being extensively studied, the function of dendritic spines is still not well understood. Below I will describe the composition of dendritic spines and discuss the different hypotheses which have been put forward to explain how they might modify synaptic transmission.

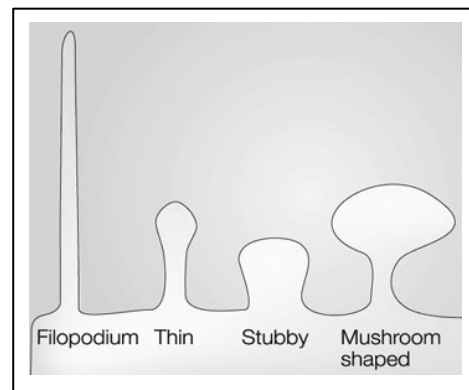


Figure 2: Schematic overview showing the four different classes of dendritic protrusions. (Adapted Hering and SHeng 2001)

1.1 Specific spine shapes are associated with distinct synapse morphologies and organelle composition:

Besides displaying a tremendous heterogeneity in shape, spines also differ in their contents of organelles and molecules. Generally speaking, large spines contain larger synapses and a greater diversity of organelles.

The postsynaptic density (PSD) is the site at the postsynapse where neurotransmitter receptors are clustered together with scaffolding and signaling molecules and can be

readily seen as a dense thickening concentrated at the surface of spines, opposite to vesicles containing presynaptic axons on electron micrographs (EM). The protein composition of PSD's consists of hundreds of components including receptors, cytoskeletal and adaptor proteins, as well as associated signaling molecules involved in several signaling pathways implicated in synaptic plasticity (Walikonis et al., 2000). Thus, the PSD can be perceived as a signal processing apparatus linked to synapse plasticity. In fixed tissue observed with EM, the PSD displays a variety of shapes ranging from simple disc structures to perforated spheres with highly irregular or segmented configurations, its dimensions being proportional to total spine volume, number of presynaptic vesicles and quantity of organelles within the spine (Chicurel and Harris, 1992; Sorra et al., 1998; Spacek and Harris, 1997). However, several studies suggest that the shape of PSD's is not set and varies with alterations in synaptic activity (Toni et al., 1999; Toni et al., 2001).

Smooth endoplasmatic reticulum (SER) is found in about half of all spines of the hippocampus and in virtually all spines of Purkinje cell of the cerebellum (Spacek and Harris, 1997). In a subset of those spines the SER elaborates into a structure called the spine apparatus, which is composed of stacks of SER laminated with dense material. The spine apparatus serves as a store for calcium, an important second messenger involved in the regulation of a variety of synaptic processes (Fifkova et al., 1983).

Coated vesicles, endosomes and multivesicular bodies are also found in spines, particularly in large spines, demonstrating that local protein degradation can occur within dendritic spines (Cooney et al., 2002; Spacek and Harris, 1997).

In addition, polyribosomes are often found in dendritic spines, indicating that protein synthesis also occurs in dendritic spines (Steward and Falk, 1985; Steward and Reeves, 1988).

The underlying cytoskeletal component which shapes dendritic spines is the actin cytoskeleton or microfilaments, while microtubules and intermediate filaments are being virtually excluded (Cohen et al., 1985; Kaech et al., 1997; Markham and Fifkova, 1986; Matus et al., 1982).

Finally, mitochondria are absent from dendritic spines but are abundant in dendrites, therefore ATP, which is essential for actin dynamics, must diffuse from the dendritic shaft into the dendritic spines (Bolan et al., 2000).

1.2 Spinogenesis:

During the first weeks of development, dendrites are covered with filopodia which are initially replaced by highly dynamic polymorphic spines and finally by the comparatively stable mature spines (Okabe et al., 2001; Takacs and Hamori, 1994; Zhang and Benson, 2000). Filopodia have a transient lifetime of minutes (Lendvai et al., 2000; Parnass et al., 2000) and sometimes bear synapses (Fiala et al., 1998). They have thus been proposed to be the precursors of dendritic spines. However it is still a matter of discussion to how mature spines could evolve from them and whether they are truly necessary for spinogenesis. Currently at least three main models explaining how spinogenesis occurs are under debate.

The first two models postulate that dendritic filopodia which are highly motile structures actively seek out a presynaptic partner (Dailey and Smith, 1996). After the filopodia has extended out of the dendrite and made an initial contact with an axon, it shortens and draws the axon towards its parent dendrite. At this stage the filopodium could either convert directly into a mature spine or alternatively, it could regress into the dendrite where it would form a shaft synapses from which a spine would grow. Two recent time-lapse studies, in which spinogenesis and PSD assembly were imaged simultaneously, showed that synapses initially form on dynamic filopodia-like spines pointing towards the first possibility (Marrs et al., 2001; Okabe et al., 2001). It thus seems that filopodia can form spines without being reabsorbed into the shaft. In contrast, other studies have shown a much higher density of synapses on dendritic shafts than on filopodia by employing serial reconstructions of EM pictures, arguing for the latter scheme (Fiala et al., 1998). Therefore both mechanisms may play a role in spinogenesis.

The third model emphasizes a constant turnover of dendritic spines during the entire lifetime of an animal, a capacity being greatest during times of enhanced plasticity, such as during critical period of circuits formation (Lendvai et al., 2000). In this scenario, dendritic spines would persistently form by seeking out presynaptic partners and stabilize into functional spines of any morphology. They could also change or even disappear depending on the quality of afferent input. With this model, the filopodium is simply a spine in an extreme state of plasticity and not a necessary stage toward spine formation. Therefore, the filopodia-to-spine transition would not be a

deterministic process, but instead one that is reversible and regulated by local factors, for example synaptic activity.

1.3 Spine pathology:

Studies from the brains of patients suffering from neurological and neuropsychiatric diseases have supplied indirect evidence for the importance of dendritic spines for normal cognition. Patients afflicted with fragile X syndrome have long and twisted dendritic spines (Hinton et al., 1991; Irwin et al., 2000; Irwin et al., 2001; Nimchinsky et al., 2001). On the contrary, in brains of individuals suffering from Down's syndrome, neurons have either very small spines or are completely absent (Marin-Padilla, 1976; Schmid et al., 1992; Suetsugu and Mehraein, 1980; Takashima et al., 1981). Alterations in spine density or shape have also been reported in patients being diagnosed with schizophrenia (Garey et al., 1998; Glantz and Lewis, 2000; Roberts et al., 1996) or Huntington disease (Ferrante et al., 1991; Ferrer et al., 1980; Guidetti et al., 2001). Therefore, the incidence of distorted dendritic spines correlates with reduced cognitive skills. However it is unclear whether spine alterations are the cause or the consequence of impaired cognition.

1.4 Significance of dendritic spine for synaptic transmission:

It has long been suggested that dendritic spine structure may modify the physiological properties of the synapses located on them. Different theoretical models have tried to clarify their mode of action (Coss and Perkel, 1985). Dendritic spines increase the interconnectivity of neurons and especially spine shape and size are suggested to influence synaptic transmission in various ways.

1.4.1 Dendritic spines increase the density of synapses and the interconnectivity between neurons:

Dendritic spines developed well before the complex mammalian brain had evolved. Neurons of simple organisms such as flatworms display dendritic spines (Keenan et al., 1981; Sarnat and Netsky, 2002) along with other invertebrate including honeybee (Brandon and Coss, 1982; Coss et al., 1980; Farris et al., 2001) and squid (Langford, 1999), who possess neurons with spine-like structures. One plausible explanation for the appearance of dendritic spines during evolution is that they are sustaining the formation of large numbers of synapses that can occur between individual neurons. In the densely packed nervous tissue, the extensions of dendritic spines provide additional surface area and therefore allow for increased synaptic density. But besides supplying a substantial increase in dendritic surface area, spines also promote interconnectivity between neurons. If one considers a two-dimensional assembly of dendrites and neurons, synapses could only be built between two adjacent neurites in dendrites without spines. Dendrites with spines can reach beyond their surface to synapse with axons in adjacent rows, thereby at least doubling the density of possible connections (Figure 3).

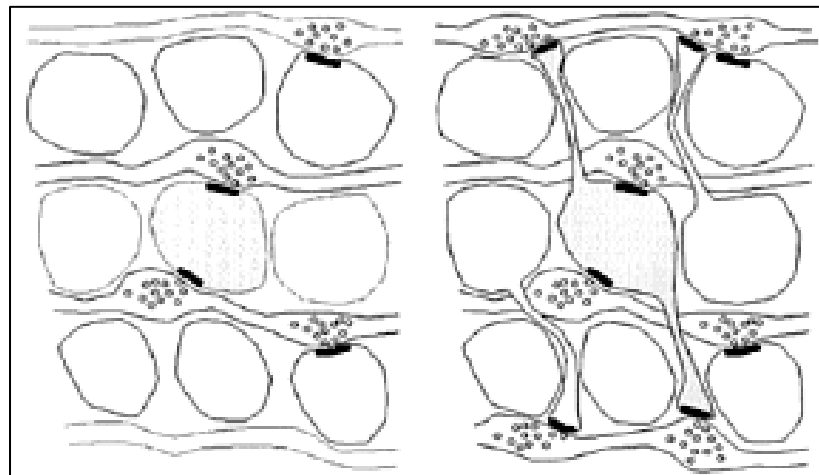


Figure 3.: Dendritic spines increase the packing density of synapses. Schematic illustration of a cross section through two dendrites (shaded), one without and one with dendritic spines. Convolution and interdigitation of dendrite, axon, and spine membranes support more synapses. (Adapted from Sorra and Harris 2000)

1.4.2 Spines could act as electrical compartments with the neck constriction amplifying membrane potential at the synapse:

Some biophysical models postulate that the spine neck can operate as a resistive module between spine head and dendritic shaft, thereby slowing charge transfer from the synapse to the parent dendrite (Segev and Rall, 1988; Segev and Rall, 1998) (Figure 4).

If this were true, changes in spine neck structure could function as a modulator of synaptic strength. However, electrical compartmental models calculated from reconstruction of serial electron microscopy pictures of spines argue against that idea (Koch and Zador, 1993; Wickens, 1988). Besides, since diffusion and electrical conduction are closely related, the range of diffusional transport between spine and dendrite has been used to estimate the electrical resistance of dendritic spine necks. These reports have also countered the concept of dendritic spines acting as electrical compartments.

Indeed, spine necks are most likely not restrictive enough to act as noteworthy hurdles for synaptic currents.

Yet, even if the resistance of a spine necks does not influence the size of current reaching the dendrite, it still could compartmentalize membrane potentials into the spine heads. This again might selectively activate voltage-dependent channels in the spine head in a manner proportional to the neck resistance and to the amplitude of the current at its synapse. If one considers the different strength of synaptic currents on individual synapses, the degree of voltage compartmentalization might differ between types of cells and synapses. Currents generated at synapses of parallel fibers on Purkinje cells, for example, are much larger than those generated on CA1 synapses by the Schaffer collateral (Barbour, 1993; Manabe et al., 1992), while the predicted average spine neck resistance is comparable in both cases (Hausser and Roth, 1997; Svoboda et al., 1996). For this reason the voltage across spine necks of CA1 pyramidal neurons is not expected to drop significantly, in contrast to the voltage

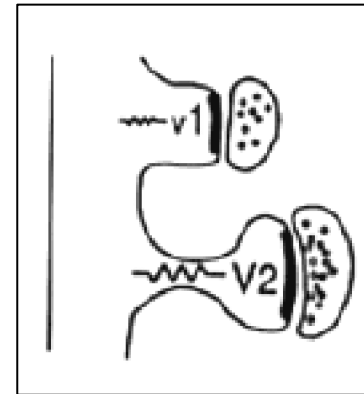


Figure 4: Spine shape and resistance of the spine neck may influence potential (V =voltage) generated by synaptic activation. (Adapted from Sorra and Harris 2000)

across Purkinje cell spine necks. Therefore electrical compartmentalization on dendritic spines may be of physiological relevance in a subset of neurons.

1.4.3 Spines act as diffusional compartments:

Transport through the spine neck can be directly assessed by measuring fluorescence recovery after photobleaching (FRAP) in individual spines (Hausser and Roth, 1997; Svoboda et al., 1996). Such studies have shown that the diffusion exchange factor between spine head and dendrite is about 100-times slower than expected for free diffusion, suggesting that dendritic spines act as biochemical compartments. This allows for the possibility that diffusional compartmentalization of second messenger or activated enzymes underlie synapse specificity in synaptic plasticity. This might be of considerable importance regarding the second messenger calcium, which besides playing a major role in regulating actin dynamics has been shown to activate various pathways that regulate synaptic transmission (Berridge, 1998). Indeed, calcium imaging studies show that spines compartmentalize calcium such that localized changes in intracellular calcium at an active synapse does not spread to neighboring inactive synapses (Guthrie et al., 1991; Muller and Connor, 1991; Yuste and Denk, 1995). It is likely that the shape and size of spines leads to variations in calcium kinetics at different synapses. These kinetic differences would then translate into different signaling events amongst individual spines. Transient calcium elevations in spines occur in consequence to two types of stimuli; orthograde synaptic stimulation and retrograde back-propagating action potentials, and are supplied by three sources; voltage sensitive or ligand-gated calcium channels which allow influx from the extracellular space, and intracellular stores. However, it is up to date not clear how an apparently omnipresent second messenger, trigger specific cellular responses. How does, for example, calcium signals control whether LTP, LTD or no changes occurs at a particular synapse? Probably, the temporal and spatial changes of intraspinal calcium transients as well as their source must be important to create specificity (Berridge, 1998).

1.5 Dendritic spine plasticity:

Spine size and shape are surely important to locally define the strength of a given synaptic input. In addition, recent live cell imaging studies have established dendritic spines as a key site of morphological plasticity in the central nervous system. Indeed dendritic spines display motility by default, as both rapid variations in spine occurring within seconds (Dunaevsky et al., 2001; Fischer et al., 1998; Star et al., 2002) and slower changes in the appearance and disappearance of individual spines over hours and days (Grutzendler et al., 2002; Lendvai et al., 2000; Trachtenberg et al., 2002) have been documented. Therefore, spines at established synapses retain a potential for morphological plasticity, in particular in response to synaptic activity (Fischer et al., 2000) or behavioral (Trachtenberg et al., 2002) as well as experimental paradigms such as LTP (Engert and Bonhoeffer, 1999), indicating that there might be a connection between changes in dendritic spine shape and experience dependent adaptation of the brain.

1.5.1 Dendritic spine motility:

The notion that dendritic spines are dynamic structures had been suggested more than two decades ago (Blomberg et al., 1977), but only the recent advances achieved in imaging tools and techniques, such as green fluorescent protein (GFP) together with the development of improved microscopes, has made it possible to address this question directly. Thanks to these advances we know now that all dendritic spines are motile by default in dissociated hippocampal cultures even after several weeks in vitro (Fischer et al., 1998) as well as in organotypic slice cultures of both the hippocampus (Fischer et al., 2000) and cerebellum (Dunaevsky et al., 2001). These observations are consistent with the high amount of actin found in dendritic spines. The involvement of actin has been confirmed by the observation that application of the actin depolymerizing drug cytochalasin D prevents rapid intrinsic spine motility. Also, it appears that actin networks rather than myosin motors are responsible for these fast shape changes since treatment with the myosin blocker BDM does not stop spine motility (Fischer et al., 1998).

Subsequently it has been shown that actin-driven spine motility is regulated by synaptic activity since application of low concentrations of AMPA or depolarization with KCl causes a rapid stop of motility and rounding up of dendritic spines both in dissociated and organotypic cultures (Fischer et al., 2000). It has also been shown that the rapid halt of motility observed after AMPA application is directly linked to the influx of extracellular calcium into spines (unpublished data). Yet another interesting finding is that volatile anesthetics such as isoflurane and chloroform, which directly act on the CNS, block dendritic spine motility, again by freezing of actin dynamics (Kaeck et al., 1999).

The finding that spines remain motile after they have established proper synaptic contacts suggests that spine motility might have other function than simple match-making or the simple contact. Those could include shifting of synapses and establishment of new contacts late into adulthood. Ultimately, if one assumes that spine motility is a requisite to synapse formation or synapse turnover and that this process continues throughout the lifetime of an organism, then one would presume that spine motility would not stop but rather decrease as the age of the organism progresses. So, is spine motility or even spine turnover truly occurring in the adult brain?

Two recent studies using two-photon microscopy to image cortical neurons visualized with GFP, have tackled this issue and generated conflicting results; one group advocating for a constant turnover of dendritic spines in the adult cortex (Trachtenberg et al., 2002) and the other monitoring an extensive stability of adult dendritic spines (Grutzendler et al., 2002). How can this discrepancy be accounted for? One possible explanation might lay in the different systems used by the two groups. Trachtenberg et al. analyzed dendritic spines from pyramidal neurons of the barrel field in the sensory cortex. The barrel field consists of tubular arrays of neurons, each array responding to stimulation of one whisker on the face of a rodent. Considering the eminent importance of whisker stimulation for rodent navigation, one would assume this system to be highly plastic. Indeed Trachtenberg and colleagues find 40% of spines to be transient; however, the majority of spines, that is 60%, remained stable during 8 days of recording under control conditions, without whisker stimulation. As will be discussed later in this chapter, sensory modulation increased spine turnover rate considerably in this system. Thus, this report suggests a constant

turnover of spines in the adult brain, modulated by sensory input. Yet, a week point of this study is the relatively young age of animals, up to 2 month maximum, being used in the experiments. Grutzendler and colleagues in contrary exploit genuinely adult mice, up to 10 months old, for their experiments. But on contrary to Trachtenberg et al., they analyzed dendritic spines on pyramidal neurons of the visual cortex. Rodents have a very poor vision and do not rely extensively on their visual system for navigation; therefore one would assume the visual cortex to be less plastic than others in rodents. In addition, mice were not subjected to new visual stimulation during the length of the experiment. Indeed, Grutzendler and colleagues find that $> 96\%$ of spines were stable over a period of three days. However, over a longer period of 4 months, only 80 % of spines remain stable using the same experimental set-up.

Thus the questions whether dendritic spines are stable or plastic in adult brain and whether mature dendrites are able to undergo substantial changes such as spine growth or loss, remains elusive. Further experiment exploiting similar experimental set-ups and truly adult animals will be required to answer the question definitely.

1.5.2 Structural plasticity induced by synaptic activity:

Under defined circumstances, dendritic spines can be selectively eliminated without apparent changes to the dendritic tree as a whole. Spine density on pyramidal neurons of the visual cortex decreased significantly after deafferentation by either contralateral enucleation or ipsilateral lesioning of the lateral geniculate, as assessed using Golgi impregnation (Globus and Scheibel, 1967). This reduction is caused by a lack of sensory input since rearing of mice in the dark also lead to a drop in spine density on pyramidal neurons of the visual cortex (Valverde, 1971), an effect which could be reversed by housing dark reared mice for a few days under normal light conditions (Valverde, 1971). In the hippocampus, spine density on dendate granule cells dropped when their main afferent axons were cut and subsequently returned to baseline levels following reafferentation by nearby axons (Parnavelas et al., 1974). These findings point towards morphological adaptation carried out by the dendrite in response to changes in afferent input. However, the concept of deafferentation-induced loss of dendritic spines cannot be applied to the entire CNS. In the weaver mutant mouse

cerebellar granule cells are lost before the formation of parallel fibers which normally synapse on spines of Purkinje cells. However, even though those cells never receive signals from their main afferent, they develop normal shapes and number of dendritic spines (Hirano, 1983; Hirano et al., 1977). Therefore it seems that dendritic spines in different parts of the brain are maintained by very different mechanisms.

Since spine synapses are glutamatergic, it might well be that glutamatergic transmission acts as a signal to maintain dendritic spines and that interfering with synaptic input might directly influence spine shape and density at least in a subset of neurons. Various studies have addressed this question and have produced quite consistent results. Increase of neuronal excitability caused by chronic blockade of GABA_A receptors, resulted in an increase in spine density on second-order dendrites in dissociated hippocampal cultures (Papa and Segal, 1996). Similarly, dendrites of neocortical organotypic cultures showed a dose-dependent increase in spine density after chronic blockade of inhibitory circuits (Annis et al., 1994). In contradiction, chronic blockade of GABA_A receptors in hippocampal organotypic slice culture had quite the opposite effect causing a dramatic loss of spines (Muller, 1993). However, this discrepancy can be easily explained by the known aberrant recurrent innervation of organotypic hippocampal cultures giving rise to epileptic activity (Gutierrez et al., 1999), which in turn is known to cause spine loss (Drakew et al., 1999; Muller et al., 1993). In yet another study blockade of AMPA receptors or blockade of vesicular release resulted in a significant decrease of dendritic spines in organotypic hippocampal cultures while blocking action potentials had no effect (McKinney et al., 1999). Thus spine maintenance requires functional AMPA receptors and moderate elevation of excitatory synaptic activity induces spine formation while its excessive increase might result in spine shrinkage caused by excitotoxicity, apoptotic neuronal cell death caused by excessive glutamate influx.

1.5.3 Dendritic spine plasticity associated with long term potentiation:

Long-term potentiation (LTP) is the observed long-lasting enhancement of synaptic strength following a brief period of high frequency stimuli. This experimental

paradigm is widely believed to underlie learning and memory (Bliss and Collingridge, 1993).

A variety of diverging spine alterations have been described following LTP induction. A first study described a specific increase in spine volume of up to 40 % on dentate granule cells following LTP stimulation of the perforant path in vivo (Van Harreveld and Fifkova, 1975). This spine enlargement decreased somewhat with time but was still evident for 23 hours after stimulation. Later the same group also observed spine shortening and neck widening using the same paradigm. Subsequently other groups supported these findings and described a 48% increase of concave spine heads and a concomitant increase in PSD area and non PSD-containing membrane following LTP. Thus LTP induction seems to alter dendritic spine shape. But how does it affect spine density which is so profoundly changed following deafferentation? A study performed by Sorra and Harris addressed that question directly by assessing spine density 2 hours after LTP induction in vivo and found no difference in spine number or density (Sorra and Harris, 1998).

However one main problem regarding the studies mentioned above is that only very few synapses are potentiated using this kind of experimental set-up and therefore there is little chance to find a stimulated synapse or even to detect associated morphological changes. More recent studies have tried to overcome this needle-in-a-haystack problem by using two-photon laser scanning microscopy to assess dendritic segments thought to be influenced by LTP stimuli based on either their proximity to the recording electrode (Maletic-Savatic et al., 1999) or because the rest of the slice was inhibited by the absence of extracellular calcium (Engert and Bonhoeffer, 1999). In both cases LTP appeared to trigger the formation of new spines. However the age of the preparations used in those studies does not allow a strict discrimination of developmentally regulated processes.

Yet another method to identify potentiated synapse was developed by Mueller and colleagues. This approach uses a histochemical procedure that marks synapses recently activated by reacting with calcium and forming an electron-dense reaction product. This method permits the analysis of synaptic features of labeled spines (Buchs et al., 1994), but not the determination of whether entirely new spines have been induced as a result of the stimulation. Nonetheless this study demonstrates a transient increase in the proportion of labeled spines with complex perforated PSD's

among activated spines relative to the general spine population (Toni et al., 1999). A longer lasting increase in the incidence of boutons forming synapses with multiple spines has also been reported using the very same approach (Toni et al., 2001).

Taken together it appears that the effect of LTP on spine morphology is not well defined. Effects are more likely local and changes in tissue are most conceivably correlated with the number of stimulated axons and dendrites, values which may vary considerably from study to study. Nonetheless there are a number of indications demonstrating a connection between LTP and changes in spine morphology or density. However, alterations in spine density or spine bifurcation are inadequate to explain the phenomenon of long term potentiation since they occur on a low time scale while the onset of LTP is a rapid process. Still, because of the suggested influence of spine shape on synaptic transmission, they could underlie, at least in part the formation of long lasting changes in synaptic strength.

1.5.4 Dendritic spine plasticity linked to experience and learning:

Does dendritic spine plasticity play a role in learning and memory? If the needle-in-a-haystack problem makes it difficult to link spine plasticity to LTP, this applies even more to learning. Even so, several studies have tried to directly link changes in spine shape to learning and have generated conflicting results. A study using confocal microscopy described an increase in spine density on basal dendrites of CA1 pyramidal neurons after rats had undergone spatial learning (Moser et al., 1994) but controversially another group using a similar approach described an increase in the clustering of synaptic zones but was unable to detect any increase in spine density (Rusakov et al., 1997). Continuing on that line another study assessing synapses in rabbit hippocampus following trace blinking conditioning, a sort of hippocampus-dependent associative learning, found an increase in PSD area but no apparent change in synapse number (Geinisman et al., 1987). O'Malley and colleagues described a transient synapse increase which subsided after 72 hours following passive avoidance training as well as spatial learning. The transient character of these changes suggests that they reflect a net rearrangement of synapses rather than a lasting increase in their number (O'Malley et al., 1998).

2 The cytoskeleton:

Eukaryotic cells contain a complex network of protein filaments that extend throughout their cytoplasm and sets up their shape. Those protein filaments are collectively termed the cytoskeleton, in analogy to the bony skeleton which determines body shape. This nomenclature is somewhat misleading since in contrary to the stiff and stable skeleton, the cytoskeleton is a highly dynamic structure. Cytoskeletal proteins control not only cell shape, but also coordinated and directed movement and are as well involved in a diversity of fundamental processes such as intracellular transport, mitosis and signal transduction.

These various actions of the cytoskeleton depend on the three types of protein filaments which will be discussed below: The intermediate filaments, the microtubules and the microfilaments or actin cytoskeleton.

2.1 Intermediate filaments:

Intermediate filaments (IF) are robust cables assembled from polymers of a large family of fibrous polypeptides and are found in the cytoplasm of most but not all animal cells. A diversity of tissue-specific forms is known that differ in the type of polypeptide residue they contain. The monomers of the different types of intermediate filaments differ substantially in amino acid sequences and molecular weight but they all contain a homologous central rod region that forms an extended coiled-coiled structure when the protein dimerize. The biological role of IF is not entirely understood but there is evidence that their main function is to resist mechanical stress (McLean and Lane, 1995).

2.2 Microtubules

Microtubules, stiff polymers of tubulin molecules, are the cytoskeletal filaments with the largest diameter. 13 protofilaments, filaments built from α and β tubulin heterodimers, align in parallel to form hollow tubules of 25 nm diameters.

Microtubules have an inherent rigidity and can therefore withstand compression and bending forces much better than any other type of protein filaments. They are polarized structures due to the head to tail arrangement of their dimeric subunits and the resulting differential behavior of the two ends, conventionally termed plus and minus ends. Microtubules exhibit a unique property called dynamic instability meaning that they go through random cycle of growth and shrinkage (Mitchison and Kirschner, 1984). At the plus end, new subunits are added in a GTP-dependent manner at a constant rate whereas the minus end stays relatively stable or even loses subunits. Hydrolysis of the bound GTP takes place after assembly and weakens the bonds that hold microtubules together. Slowly growing microtubules are especially susceptible to catastrophic disassembly but can be stabilize by association with other structures that cap their two ends. Within the cytoplasm, microtubules are anchored at an organizing center associated with the centrosomes, which protects the minus end of microtubules and continually nucleate the formation of new microtubules (McNally, 1996). Any microtubule that encounters a structure that stabilizes its free plus end will be selectively retained whereas other microtubule will depolymerize, a process held largely responsible for the positioning of microtubule arrays in a cell. Selectively stabilized microtubule are eventually acetylated or detyrosinated, a process providing sites for the binding of specific microtubule-associated proteins (MAP's), which further stabilize the microtubule against disassembly. Microtubule motor proteins, dyneins and kinesins, constitute an important class of MAP's that use the energy of ATP hydrolysis to move unidirectionally along a microtubule carrying specific cargo. Such proteins are largely responsible for the spatial organization and directed movements of organelles in the cytoplasm.

2.3 Actin:

Actin filaments or F-actin, are thin, flexible filaments. They consist of a tight helix of uniformly oriented actin (globular or G-actin) molecules. Like microtubules, actin filaments are polarized structures, with two structurally different ends; a relatively inert and slow-growing minus-end (also called the pointed end) and a faster growing plus-end (also called the barbed end). In mammals there are at least six actin isoforms

known which fall into three classes based on their biophysical properties. The α isoforms are important components in various types of muscles, whereas β and γ isoforms are the principal constituents of nonmuscle cells. Although there are subtle differences in the properties of different forms of actin their amino-acid composition has been highly conserved through evolution and all assemble into filaments that are essentially identical in most tests performed *in vitro*. Polymerization of pure G-actin to F-actin *in vitro* requires ATP as well as both monovalent and divalent cations.

Varieties of structures, ranging from stiff and rather stable extensions from the cell surface to dynamic networks at the leading edge of migrating cells, are based on the actin cytoskeleton and can coexist in a living cell. In every case the elementary configuration of the actin filament is the same. What varies in these different cytoskeletal assemblies are the length, the stability and the strength of the individual actin filament as well as the number and pattern of inter-filament attachments. These properties in turn depend on a large train of actin-binding proteins of which a few will be discussed below.

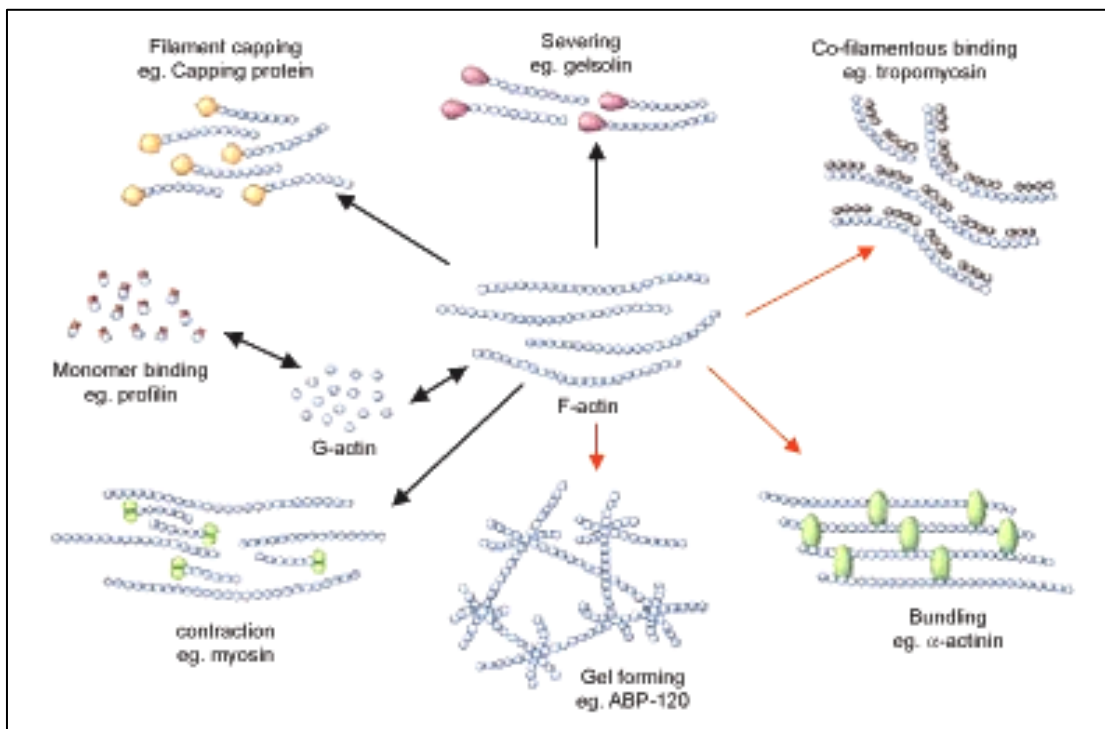


Figure 5: Functions of actin-binding proteins determined from *in vitro* research. Many actin-binding proteins have been purified and their properties and effects on actin have been extensively studied. How these *in vitro* functions relate to the role of the actin-binding proteins within a cellular environment remains largely unknown. Specific functions of actin-binding proteins are shown with a diagram of how each protein may interact with F-actin. Examples of proteins that may fulfill these functions are also given.

2.3.1 Actin binding proteins

2.3.1.1 Actin binding proteins affecting the polymerization state of actin:

The rapid reorganization of the actin cytoskeleton, essential for directed movements of cells require the action of proteins that affect actin polymerization kinetics. The initiation of actin polymerization, or nucleation, is a rather slow process when carried out in vitro. Nevertheless, once nucleation has occurred filament elongation proceeds rapidly until a steady state between the G- and F-actin pools is reached. Therefore, proteins which initiate terminate, or affect in any other way the kinetics of actin polymerization, are key regulators of actin dynamics in vivo.

The Arp2/3 complex and its regulators, Scar and Wasp:

The Arp2/3 complex is a stable assembly of seven proteins which is found in all eukaryotic cells. It functions as initiator of new barbed ends by creating branches at or near the end of existing actin filaments (Pollard and Beltzner, 2002). Activation of Arp2/3 and initiation of actin elongation depends on extrinsic regulators which were identified as the WASp/Scar proteins using yeast two hybrid screens. The C-terminal VCA domain of WASp/Scar proteins consists of a verprolin homology domain (also called VASp homology or VH-2 domain) that binds actin monomers, a terminal acidic domain, A, that binds the Arp2/3 complex and a connecting domain C and is responsible for activating Arp2/3. In the resting state WASp/Scar proteins are folded and the VCA domain is masked. Upon binding to PIP₂ and cdc42 the proteins unfolds so that the VCA domain is exposed and can bind to actin and Arp2/3. Binding of WASp/Scar proteins to actin and the Arp2/3 complex is cooperative (Marchand et al., 2001). Because of the crucial role of PIP₂ and cdc42 in Arp2/3 activation, Arp2/3 mediated actin nucleation and branching is particularly associated to the leading edge of migrating cells where it directs

Actin capping and severing proteins:**The cofilin/ADF family**

The members of the cofilin/ADF family are small actin severing proteins that promote depolymerization of actin filaments in a pH-dependent manner (Mitchison and Kirschner, 1984). Eukaryotic cells express at least one member of the family, suggesting a fundamental role for cofilin/ADF in vivo. Supporting this notion, it has been shown that null mutations in cofilin/ADF genes are lethal in various organisms (Aizawa et al., 1995; McKim et al., 1994; Moon et al., 1993). Using partial loss-of-function alleles in the yeast cofilin gene, Lappalainen and colleagues (Lappalainen and Drubin, 1997; Lappalainen et al., 1997) demonstrated that rapid actin assembly and disassembly depend on cofilin. Cofilin and ADF are both negatively regulated by phosphorylation by LIM kinase, which dramatically decreases its ability to interact with F-actin (Arber et al., 1998).

The gelsolin family

The members of the gelsolin family of actin-binding proteins sever and cap the barbed ends of actin filaments in a Ca^{2+} dependent manner. Phosphatidylinositol-4,5-bisphosphate (PIP₂), in contrary, inhibits gelsolin activity by causing its dissociation from actin (Weeds and Maciver, 1993; Weeds et al., 1993). Gelsolin has been implicated in mediating the rapid motile responses in cell types involved in stress responses such as hemostasis, inflammation and wound healing (Witke et al., 1995). Moreover, overexpression of gelsolin causes an increase in fibroblast cell motility (Cunningham et al., 1991) which requires the severing activity of gelsolin (Arora and McCulloch, 1996).

2.3.1.2 Actin binding proteins involved in stabilizing filaments and in generating supramolecular structures:

Many proteins that bind to the actin cytoskeleton impede its dynamic behavior. These proteins stabilize filaments or link filament in manifold ways to produce higher order structures.

Side binding proteins:

The most widespread members of side-binding proteins are the tropomyosins, rigid rodlike proteins that bind along actin filaments, spanning up to seven actin monomers (Pittenger et al., 1994). They have been found in a wide range of cell types, including yeast (Balasubramanian et al., 1992), suggesting that they play a fundamental role for the regulation of the actin cytoskeleton. Tropomyosin-bound actin filaments are stiff and rigid. Most cells contain several isoforms of tropomyosins which differ in sequence and molecular weights. High molecular weights tropomyosins associate particularly well with actin filaments and are abundant in stable structures such as stress fibers.

Actin cross-linking proteins:

In animal cells the cortical actin filaments are organized into three general types of arrays; in parallel bundles as seen in microspike, in contractile bundles as found in stress fibers or as gel-like network, where filaments are arranged in a loose, open array with many crossed interconnections.

Actin filament cross-linking proteins can be divided into two classes, bundling proteins and gel-forming proteins according to their effects on pure actin filaments in vitro. Two widely distributed cross-linking proteins are fimbrin and α -actinin. Fimbrin organizes F-actin in straight parallel bundles at the leading edge of cells especially in microspikes or filopodia whereas α -actinin packs F-actin into loose antiparallel bundles. Filamin is a widely distributed gel-forming protein that promotes the formation of a loose and highly viscous network by clamping together two actin filaments that cross each other. These proteins direct crosslinking and bundling by utilizing two discrete actin binding sites. The distance separating these two sites is an important determinant of the type of higher order F-actin structure formed. Members of the largest class of actin crosslinking proteins, including fimbrin, α -actinin, dystrophin and spectrin are defined by a conserved 27 kDa F-actin binding domain. This actin binding domain (ABD) spans approximately 250 amino acids and is composed of tandem repeats of two calponin homology (CH) domain. In addition to directing the specific packing properties of actin bundles and networks, modular

organization affords a level of regulation essential for biological function. Once crosslinked assemblies are formed, they may be kinetically stable. This stability is due to high local concentration of both crosslinkers and actin filaments in these structures. Therefore, changes in their concentrations alone may not be sufficient to elicit the rapid assembly or disassembly of these structures that is required for the dynamic processes in which they are involved.

Drebrin:

Drebrin, (Devopmentally regulated brain protein), is an F-actin binding protein originally identified in chick brain (Ishikawa et al., 1994). Meanwhile, drebrin has been found in a variety of cell-types and tissues (Keon et al., 2000; Luna et al., 1997; Peitsch et al., 1999; Peitsch et al., 2001).

Although the function of drebrin is poorly understood; drebrin has no severing, nucleating or cross-linking activity (Ishikawa et al., 1994), several lines of evidence from biochemical and cell culture studies support a general involvement for this protein in controlling the arrangement of stable actin arrays. Experiments with non-neuronal cells have demonstrated that drebrin competes for binding to F-actin with α -actinin, fascin and tropomyosin, proteins involved in the formation and stabilization of actin filament networks. In biochemical tests drebrin has been found to displace α -actinin, tropomyosin (Ishikawa et al., 1994) or fascin (Sasaki et al., 1996) from actin filaments assembled in vitro. Moreover over-expressed drebrin produces irregular cell extensions in cultured non-neuronal cells (Hayashi et al., 1999) and displaces tropomyosin from actin filaments leading to its subsequent down-regulation in CHO cells (Ishikawa et al., 1994). Furthermore, drebrin has been shown to inhibit acto-myosin contraction (Ishikawa et al., 1994). In addition to its ability to interact with F-actin, drebrin is coimmunoprecipitated in a stable complex together with gelsolin, α -actinin and myosin from rat brain (Hayashi et al., 1996), and interacts directly with profilin as seen in affinity binding studies (Mammoto et al., 1998).

Adherens junctions and stable anchorage of actin filaments:

The cortical layers of most tissue cells are variegated. Many of them possess specialized regions in which bundles of actin are attached to the plasma membrane through specific sets of associated proteins. Some of them are responsible for cell-cell anchorage whereas others attach the cell to its substratum. Many adult tissues are held together by adherens junctions, which connect actin filaments in adjacent cells (Gumbiner, 1996). In non-epithelial cells these usually take the form of small punctae or streaklike attachments whereas in epithelial cells they form a continuous adhesion belt or zonula adherens around the apical end of the cell. The apposing faces of an adherens junction are held together by membrane proteins capable of self association, principally the family of Ca^{2+} dependent cells adhesion molecules, the cadherins (Braga, 2000). These span the plasma membrane having a cytoplasmic domain that becomes associated to the cytoskeleton via adaptor proteins, the catenins (Petzelbauer et al., 2000).

Focal contacts (or adhesion plaques) are the site in a cell where actin filaments are anchored to the extracellular matrix. Formation of focal contacts depends on the cell substratum and is typically promoted by two proteins found in the serum, fibronectin and vitronectin. The cell receptors involved in the formation of focal contacts formation belong to the large family of integrins (Critchley, 2000; Parsons et al., 2000). An integrin molecule is a non-covalently associated complex of two distinct, high molecular weight polypeptides, called α and β integrins that act as transmembrane linker in a variety of cells. Integrins span the plasma membrane in the region of focal contacts. Its cytoplasmic domain interacts with talin which in turns binds to vinculin. Vinculin interacts with α -actinin which in turns binds to actin filaments therefore providing the link between adhesion complexes and the cytoskeleton (Critchley, 2000).

2.3.2 Regulation of actin by small GTP-ases:

Small GTPases operate as central molecular switches, cycling between an inactive GDP-bound state and an active GTP-bound state. The ratio of the two states is regulated by the opposing effects of guanine nucleotide exchange factors (GEF's), which enhance the exchange of bound GDP for GTP, and the GTPase activating proteins (GAP's), which increase the intrinsic rate of hydrolysis of bound GTP. Members of the Ras superfamily of GTPases, including Ras and the members of the Rho superfamily of GTPases appear to be key players in controlling the organization of the actin cytoskeleton. Rho controls the formation of stress fibers and focal adhesion complexes, Rac regulates actin filaments accumulation at the plasma membrane to produce lamellipodia and membrane ruffles and cdc42, triggers the formation of filopodia (Hall, 1998).

The initial work of Hall and colleagues established Rho as a key regulator controlling the assembly of stress fibers and focal adhesion complexes (Hall, 1998). Rho activates ROCK, which phosphorylates and thereby inactivates myosin light chain (MLC) resulting in activation of myosin contractility (Kimura et al., 1996). This activation appears to be a critical step for the formation of focal adhesions since pharmacological agents that block actomyosin contractility, inhibit Rho-induced stress fiber and focal adhesion formation (Chrzanowska-Wodnicka and Burridge, 1996).

Ras-transformation of cells in tissue culture results in a variety of cellular changes including alteration in serum- and adhesion dependent cell growth, loss of contact inhibition, changes in adhesiveness, motility and morphology. Anchorage independent growth and tumorigenicity, suggesting a fundamental role for actin filaments networks in oncogenic transformation. Indeed, the alterations in actin filament structure observed in transformed cells were found to correlate with decreased expression in several cytoskeletal proteins and re-expression of these proteins, including α -actinin, gelsolin (Mullauer et al., 1990) and tropomyosin (Gimona et al., 1996), suppresses transformation. Ras itself regulates a signal transduction pathway linking plasma membrane receptors to the ERK MAP kinase

cascade, an essential and rate limiting signal for growth and differentiation. However, besides controlling gene expression, the MAPK pathway has also been directly involved in regulating cytoskeletal structures. ERK interacts directly with α -actinin and is translocated to stress-fibers (Leinweber et al., 1999). Furthermore, the MAPK pathway activates caldesmon which in turn inhibits acto-myosin contraction (Agell et al., 2002; Pritchard and Moody, 1986)

The Ras effector MEKK1 activates calpain, which subsequently cleaves adhesion complexes at the trailing edge of migrating fibroblasts (Cuevas et al., 2003).

2.4 The neuronal cytoskeleton:

If one examines the distribution of the three basic cytoskeletal elements in neurons, the following picture emerges: the neuron-specific IF and microtubules are restricted to neurite shafts where they are associated to tau in axons and MAP-1 and MAP-2 in dendrites and therefore very stable.

As stated earlier, the cytoskeleton underlying dendritic spines is the actin cytoskeleton. Both microtubules and intermediate filaments are excluded from this structure (Kaech et al., 2001). The two actin isoforms expressed in neurons are the β and the γ isoforms which are both selectively targeted to spines by an unknown mechanism (Kaech et al., 1997).

Dendritic spines are surrounded by a motile corona (Fischer et al., 1998; Star et al., 2002), which changes shape on a time scale of second, due to dynamic actin polymerization as motility is blocked by the actin depolymerizing drug cytochalasin D but not the myosin light chain inhibitor BMD (Fischer et al., 1998). This highly motile corona is superimposed on a very stable core actin network, which is resistant to treatment with cytochalasin D and can only be disassembled by extended treatment with latrunculin A, pointing towards a slow actin turnover of this structure (Allison et al., 2000). These findings are consistent with early electron microscopy studies which indicated a partitioning of actin filaments within the spine cytoskeleton with long filament bundles extending from the spine neck into the core of the head region

whereas the head periphery contains irregularly oriented small bundles and single filaments (Cohen et al., 1985; Landis and Reese, 1983).

AIM OF THIS THESIS:

Dendritic spines are small protrusions on which most excitatory synapses in the brain are made.

Dendritic spines can modulate synaptic transmission in various ways and have in addition been shown to be highly motile, displaying both rapid surface motility and slower structural plasticity (see General introduction).

Both, the morphology and the plasticity of dendritic spines is likely to be governed by filamentous actin since other cytoskeletal components, such as microtubules, are absent from these structures. Currently little is known about the molecular components that regulate actin cytoskeletal function within dendritic spines.

The aim of this thesis was to identify cytoskeletal components implicated in the regulation of morphological plasticity at the synapses.

Part I**DISTRIBUTION OF ACTIN BINDING PROTEINS IN MATURE
HIPPOCAMPAL NEURONS**

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SUMMARY

The morphology and plasticity of dendritic spines, the major sites of excitatory synapses in the brain, is likely to be governed by filamentous actin since other cytoskeletal components, such as microtubules, are absent from these structures. Currently little is known about the molecular components that regulate actin cytoskeleton function within dendritic spines.

We have examined the subcellular distribution of several actin binding proteins which have formerly been implicated in actin dynamics or in the stabilization and anchorage of F-actin in non-neuronal cells, in dendritic spines.

We demonstrate that molecules involved in fast actin treadmilling, the Arp2/3 complex, cofilin and gelsolin, are components of dendritic spines. Furthermore we confirm that the actin crosslinking protein α -actinin, and that drebrin, a protein impeding the formation and maintenance of bundled actin are localized with dendritic spines.

Cell-cell adhesion appears to be restricted to the postsynaptic spines within our culture system since β -catenin was located exclusively to dendritic spines. Finally, a general cell adhesion molecule, vinculin was distributed diffusely throughout the cytoplasm of dissociated neurons.

Together these observations suggest that the influence of actin dynamics on dendritic spine structure and motility is regulated by the same major actin binding proteins as are involved in controlling surface motility and morphogenesis in non-neuronal cells. The central issue, therefore, in the control of synaptic plasticity of central nervous synapses is the nature of the coupling between neurotransmitter receptors and neuromodulators and the activation of the same actin regulating proteins.

INTRODUCTION

Dendritic spines, consisting of a bulbous head connected to the dendritic shaft by a narrow neck, are the primary site of excitatory synapses in the adult brain.

Spines are thought to influence synaptic transmission in several ways. Through their sheer presence, they increase the density of synapses and the interconnectivity between neurons and are moreover believed to act as electrical compartments, amplifying membrane potentials at the synapse. In addition, dendritic spines necks prevent free diffusion of second messenger and activated enzyme to the parent dendrite, spine heads therefore operating as biochemical compartments (Hausser and Roth, 1997; Svoboda et al., 1996). (Chang and Greenough, 1984; Peters and Kaiserman-Abramof, 1970) All these alleged functions are conceivably to be influenced by the specific shape a particular dendritic spine adopts, which is likely to modulate the type and the extent of modifications applied to synaptic currents before they reach the parent dendrite.

Based on early ultrastructural analysis, dendritic spines have been arbitrarily classified into three categories according to their overall morphologies; thin spines, with a small head and relatively long neck, mushroom-type spine with a large head and stubby spines which basically lack any neck (Chang and Greenough, 1984; Peters and Kaiserman-Abramof, 1970). However, this classification misleads towards a static view of spine morphology. In fact, dendritic spines exhibit rapid shape changes within seconds, which are blocked by glutamate receptor activation (Fischer et al., 1998; Fischer et al., 2000; Star et al., 2002). Moreover, on longer time scales, dendritic spine can experience a variety of structural modifications, ranging from growth to collapse or elongation to shortening (Grutzendler et al., 2002; Lendvai et al., 2000; Trachtenberg et al., 2002).

Regarding the proclaimed importance of dendritic spine shapes for synaptic transmission, it is a great concern to understand how spine morphology, motility and structural stability or plasticity is regulated. As filamentous actin is the principal cytoskeletal element in dendritic spines (Kaech et al., 1997; Matus et al., 1982) and is therefore likely to govern these processes.

Actin filaments have been implicated in a diversity of cell processes, ranging from intracellular transport (Ayscough, 2000; Smythe and Ayscough, 2003) to signaling (Schell et al., 2001) in a variety of cells and are of major importance for directed cell movement (Higgs and Pollard, 2001; Pollard and Borisy, 2003). These central tasks performed by the actin cytoskeleton are regulated by a train of actin binding proteins (Ampe and Vandekerckhove, 1994). In recent years major advance has been made in regard to understanding how some of these proteins regulate the various functions carried out by the actin cytoskeleton. Yet, the composition and the organization of the actin cytoskeleton in dendritic spines are still not well understood and up to date only a few actin binding proteins have been reported in dendritic spines (Smart and Halpain, 2000).

Dendritic spines are surrounded by a motile corona (Fischer et al., 1998; Star et al., 2002), which changes shape on a time scale of second, due to dynamic actin polymerization as motility is blocked by the actin depolymerizing drug cytochalasin D but not the myosin light chain inhibitor BMD (Fischer et al., 1998). This highly motile corona is superimposed on a very stable core actin network, which is resistant to treatment with cytochalasin D and can only be disassembled by extended treatment with latrunculin A, pointing towards a slow actin turnover of this structure (Allison et al., 2000).

The molecular mechanisms underlying rapid actin dynamics have been extensively studied in the leading edge of migrating fibroblasts and a diversity of actin binding proteins which governs fast actin treadmilling have been identified (Pollard and Borisy, 2003). In this study we used hippocampal dispersed neurons to identify the subcellular distribution of three of these molecules, the Arp2/3 complex (Amann and Pollard, 2001; May et al., 1999), cofilin (Blanchoin et al., 2000) and gelsolin (Ayscough, 1998), and their relation to dendritic spines. We find that proteins promoting fast actin turnover are major components of dendritic spines actin. The two severing and capping proteins cofilin and gelsolin are key components of the actin cytoskeleton dendritic spines, expressed in virtually every spine while the Arp2/3 complex was absent from a subset of spines.

Slow actin turnover, in contrary, is generally associated to bundled or cross-linked actin (Blanchoin et al., 2001) anchored to neighboring cells or extracellular space.

We therefore studied the distribution of two proteins regulating the formation and maintenance of stable actin networks; the cross-linking protein α -actinin (Xu et al., 1998), which is also implicated in the anchoring of actin filaments to adhesion complexes (Gilmore and Burridge, 1996) and drebrin, a protein which impedes actin bundling by competing with fascin (Sasaki et al., 1996), tropomyosin and α -actinin (Ishikawa et al., 1994). We confirmed the localization of these molecules in dendritic spines and observed a close association of drebrin with those structures, arguing for an important role of stable actin filaments and their regulation for shaping the postsynapse.

Together these observations suggest that the influence of actin dynamics on dendritic spine structure and motility is regulated by the same major actin binding proteins as are involved in controlling surface motility and morphogenesis in non-neuronal cells.

The central issue, therefore, in the control of synaptic plasticity of central nervous synapses is the nature of the coupling between neurotransmitter receptors and neuromodulators and the activation of the same actin regulating proteins.

RESULTS

The Arp 2/3 complex, cofilin and gelsolin, which promote actin dynamics, are present in dendritic spines:

To examine the molecular composition of the actin cytoskeleton of dendritic spines we transfected dispersed hippocampal neurons with GFP-actin to make dendritic spines readily visible (Fischer et al., 1998). Neurons were fixed after three weeks of culture, when they have fully developed and established mature synapses in vitro (Kaech et al., 2001), and were subsequently stained with various antibodies directed against different actin binding proteins. Successive analysis was performed using confocal microscopy and three-dimensional reconstruction.

We first looked at the relative distribution of spine subtypes on dendrites in our culture system by counting thin, mushroom type or stubby spines per 100 μm portions on dendrite of 30 individual neurons. The majority, accounting for $66 \pm 15.1\%$ of all dendritic spines in our culture system, were mushroom shape spines, characterized by a large bulbous head connected to the dendritic shaft by a narrow neck. The other two classes of dendritic spines as to say thin spines distinguished by a small head connected by a thin neck and to the dendritic shaft and stubby spines, exhibiting a bulbous head directly connected to the shaft, were distributed in similar amounts on dendrites; $17.8 \pm 8.6\%$ of protrusion were stubby spines while $15.9 \pm 11.2\%$ were thin spines.

To determine the potential role of some of the most prominent proteins involved in actin treadmilling at the leading edge, the Arp2/3 complex, cofilin and gelsolin in modulating rapid intrinsic spine motility or spine morphology, we studied their expression and subcellular localization in mature dispersed hippocampal neurons.

The first candidate molecule we chose to survey was the Arp2/3 complex, a protein assembly composed of seven proteins, known to play a key and essential role in actin nucleation and branching at the leading edge of migrating fibroblasts (Amann and Pollard, 2001; May et al., 1999). To assess the distribution of the complex in mature neurons, we stained cultures with two antibodies directed against two different

proteins of the complex, p-34-arc and arp3 (Robinson et al., 2001). When stained with either antibody, we find that the Arp2/3 complex is expressed in mature dispersed hippocampal neurons, where it is distributed in a distinct punctuate pattern (Figure 1A). At higher magnification it became evident, that p-34-arc and arp3 punctae were distributed around dendrites (Figure 1B'), which often were strongly colocalized with dendritic spines visualized by GFP-actin (Figure 1B, B' and C, closed arrow). The majority of dendritic spines were Arp2/3 positive (Figure 1B, B' and C, closed arrows), however, a significant fraction of dendritic spines visualized by GFP-actin did not colocalize with Arp2/3 punctae (Figure 1B, B' and C, open arrows). When this observation was quantified by counting Arp2/3 positive spines on 100µm portions of 20 individual neurons (10 stained with arp3 and 10 stained with p-34-arc) we found $81.9 \pm 8.0\%$ of spines to express the Arp2/3 complex and $18.1 \pm 8.0\%$ of spines to bear any Arp2/3 signal.

Yet, the relative contents of Arp2/3 complex within a spine could not be attributed to a particular spine subtype. Indeed Arp2/3 signal could be detected in thin, stubby and mushroom shaped spines or was absent of them indiscriminately. The observations stated above were substantiated by counting stubby, thin or mushroom-type spines showing Arp2/3 staining in 100 µm portions of dendrites of 20 individual stained neurons. On average $79.6 \pm 18.0\%$ of stubby, $81.8 \pm 39.6\%$ of thin and $81.9 \pm 14.4\%$ of mushroom-type spines showed detectable Arp2/3 signal.

Therefore, it appears that the Arp2/3 complex is a major but not an essential constituent of the actin cytoskeleton in dendritic spines. Moreover, Arp2/3 complex expression is not linked to a specific spine structure.

Another protein involved in fast actin turnover at the leading edge of migrating fibroblasts is ADF/cofilin which governs actin treadmilling by severing and depolymerizing actin filaments (Pollard and Borisy, 2003) and is negatively regulated by LIM kinase (Blanchoin et al., 2000). We surveyed cofilin expression in adult dispersed neurons by immunostaining GFP-actin transfected neurons with a cofilin specific antibody.

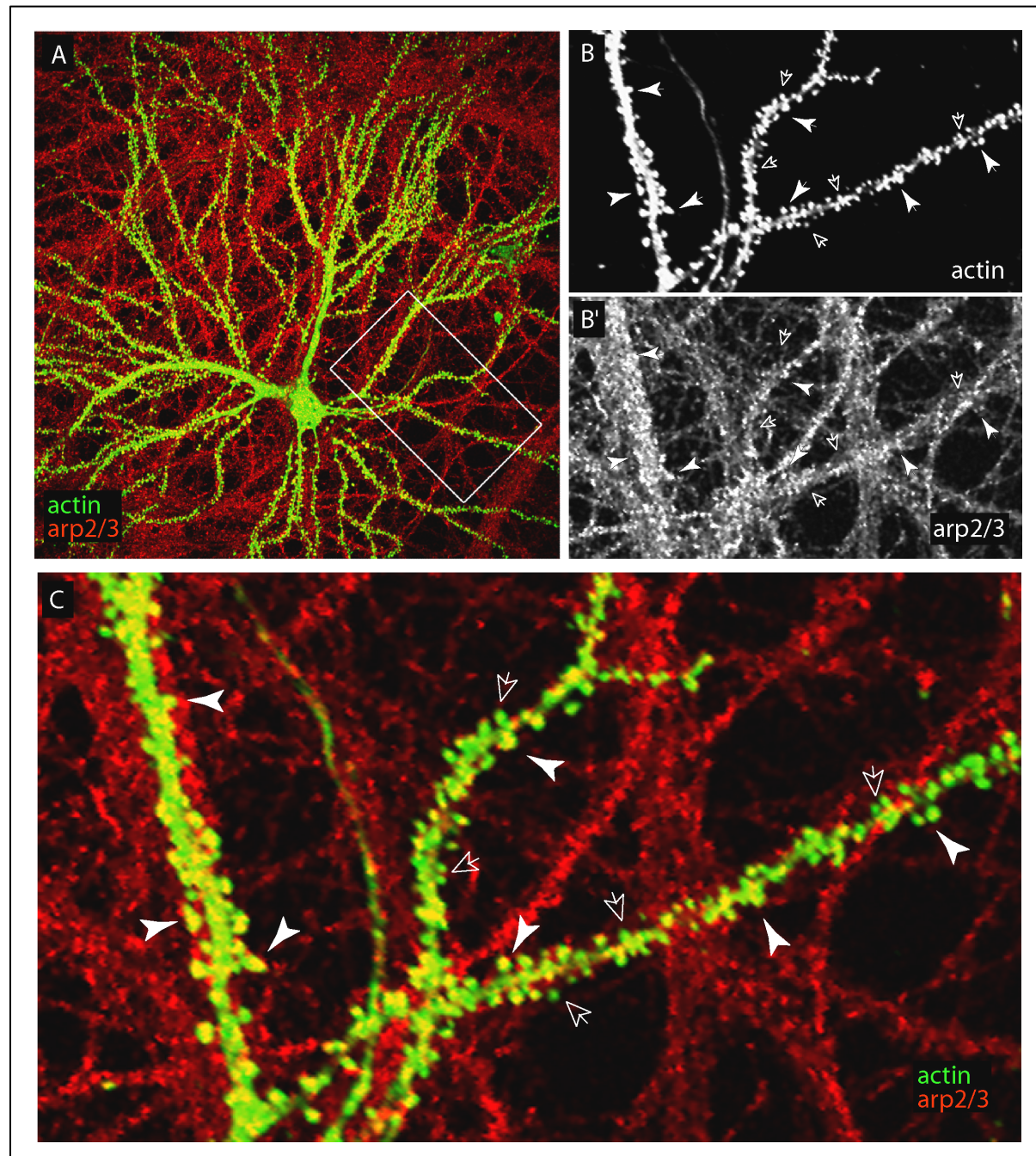


Figure 1: The arp2/3 complex is concentrated in a majority of postsynaptic spines

(A) Confocal image from a mature neuron transfected with GFP-actin (green) and counterstained with an antibody directed against p-34-arc (red), a component of the arp2/3 complex. The Arp2/3 complex is expressed in mature neurons and displays a punctuate staining pattern. (B-C) Magnified images of area designated in A. (B) Dendritic segment designated in A, visualized by GFP-actin. Dendrites grow spines of every category, stubby, thin and mushroom type (closed arrowheads and open arrows) (B') Arp2/3 complex visualized by p-34 arc immunoreactivity in area designated in A. The Arp2/3 complex is enriched in punctae along the less strongly stained dendritic shaft (closed arrowheads and open arrows). (C) Merge from B and B' (GFP-actin=green, arp2/3-complex=red). Note that the arp2/3 complex is enriched in a majority (closed arrowheads) but not in all dendritic spines (open arrows)

Cofilin, as the Arp2/3 complex, is expressed in adult neurons where it also shows a punctuate distribution pattern (Figure 2A). At higher magnification it is clearly visible that the majority of cofilin punctae colocalize with dendritic spines (Figure 2B, B' and C, closed arrowheads). When evaluated by counting cofilin in 100 μm segments of dendrites from 20 individual neurons, we find that cofilin is expressed in 100% of dendritic spines. Therefore it appears that cofilin, in contrast to the Arp2/3 complex, is a fundamental component of the actin cytoskeleton in dendritic spines.

We next looked at the distribution of another actin capping and severing protein gelsolin (Arora and McCulloch, 1996) in adult neurons. As for the arp2/3 complex and cofilin, gelsolin is expressed in adult dispersed hippocampal neurons where it also displays punctuate staining pattern (Figure 3A). Gelsolin immunoreactivity was much stronger in dendritic spines as compared to the dendritic shaft (Figure 3B, B' and C). Also, gelsolin is localized in every visible spine and appears therefore to be a core component of dendritic spine actin.

Taken together these result argues for a similar regulation machinery controlling fast actin treadmilling in dendritic spines and at the leading edge of migrating fibroblasts.

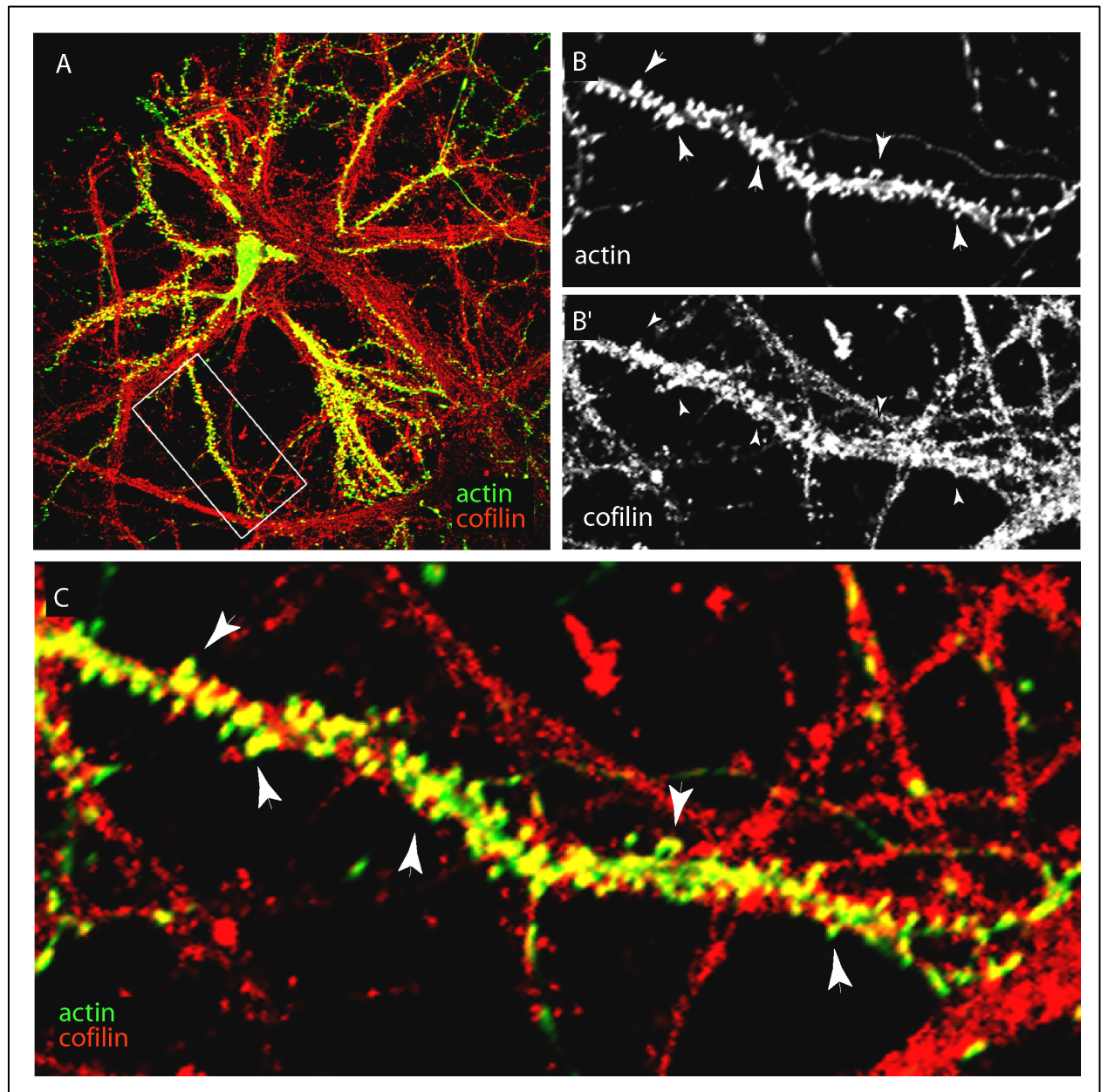


Figure 2: Cofilin is expressed in all dendritic spines

(A) Confocal image taken from a mature neuron transfected with GFP-actin (green) and counterstained with an antibody directed against cofilin (red). Cofilin is expressed in mature neurons and displays punctuate staining pattern. (B-C) Magnified images of area designated in A. (B) Dendritic segment designated in A, visualized by GFP-actin. (B') Cofilin visualized by immunoreactivity in area designated in A. Cofilin staining appears in spots surrounding the dendritic shaft. (C) Merge from B and B' (GFP-actin=green, cofilin=red). Note that the cofilin is enriched in every single spine along the designated dendrite (closed arrowheads)

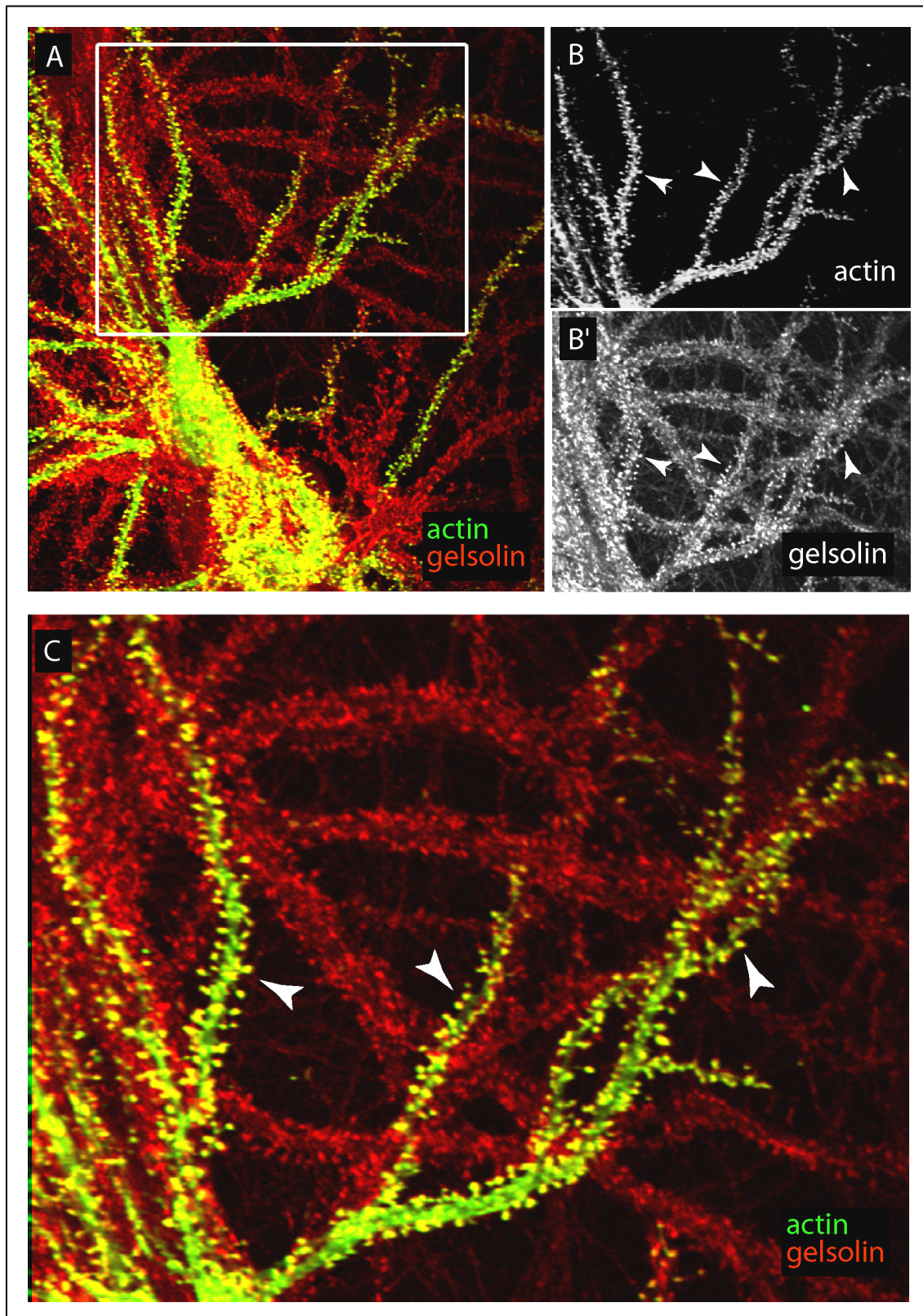


Figure 3: Gelsolin is a major component of dendritic spines

(A) Confocal image from a mature neuron transfected with GFP-actin (green) and counterstained with an antibody directed against gelsolin (red). (B-C) Magnified images of area designated in A. (B) Dendritic segment designated in A, visualized by GFP-actin. (B') Gelsolin visualized by immunoreactivity in area designated in A. Gelsolin is enriched in spots surrounding the dendritic shaft. (D) Merge from B and B' (GFP-actin=green, gelsolin=red). Note that every single spine visualized by GFP-actin contains gelsolin.

Distribution of proteins involved in the generation of supramolecular structures in dendritic spines:

Proteins regulating rapid actin treadmilling, such as the Arp2/3 complex, cofilin and gelsolin are localized to dendritic spines. What about the distribution of actin binding proteins implicated into the formation and maintenance of more rigid actin networks?

We first looked at the distribution of the actin crosslinking protein α -actinin, which stabilizes actin filaments by cross-linking them and anchoring them to cell adhesion complexes and had previously been reported to be present in dendritic spines (Allison et al., 2000; Wyszynski et al., 1998). In agreement with those previous reports we find α -actinin to be expressed in adult neurons (Figure 4A) and to be enriched in dendritic spines (Figure 4B, B' and C). However, even though α -actinin appears to be a constituent of a majority of dendritic spines it is not expressed in all of them. In total, 89.4 ± 6.3 % of all spines had detectable amounts of α -actinin, while 10.6 ± 6.3 % of all spines was α -actinin negative.

As for the Arp2/3 complex, relative content of α -actinin in a spine could not to be assigned to any particular spine morphology. The majority of mushroom-type spines, the most abundant class of spines in our culture system, for example, generally showed high content in α -actinin (Figure 4B, B' and C, closed arrowheads), however, some completely lacked α -actinin signals (Figure 4B, B' and C, open arrows). We quantified this observation by counting spines containing α -actinin in 100 μ m portions of dendrites from 20 neurons. These counts revealed that 89.4 ± 6.3 % of all spines were α -actinin positive while 10.6 ± 6.3 % lacked any signal.

Thus it appears that α -actinin is a major but not an essential constituent of the actin cytoskeleton in dendritic spines.

Drebrin, a protein associated with the regulation of binding of cross linking actin binding proteins to actin (Ishikawa et al., 1994) has also been previously reported to be enriched in dendritic spines (Hayashi et al., 1996). In agreement with these report we also find drebrin to be expressed in dispersed hippocampal neurons and to be closely associated to dendritic spines in dispersed hippocampal neurons. Indeed drebrin was expressed in every spine and was almost excluded from the dendritic shaft, implying that drebrin is a crucial constituent of dendritic spine actin.

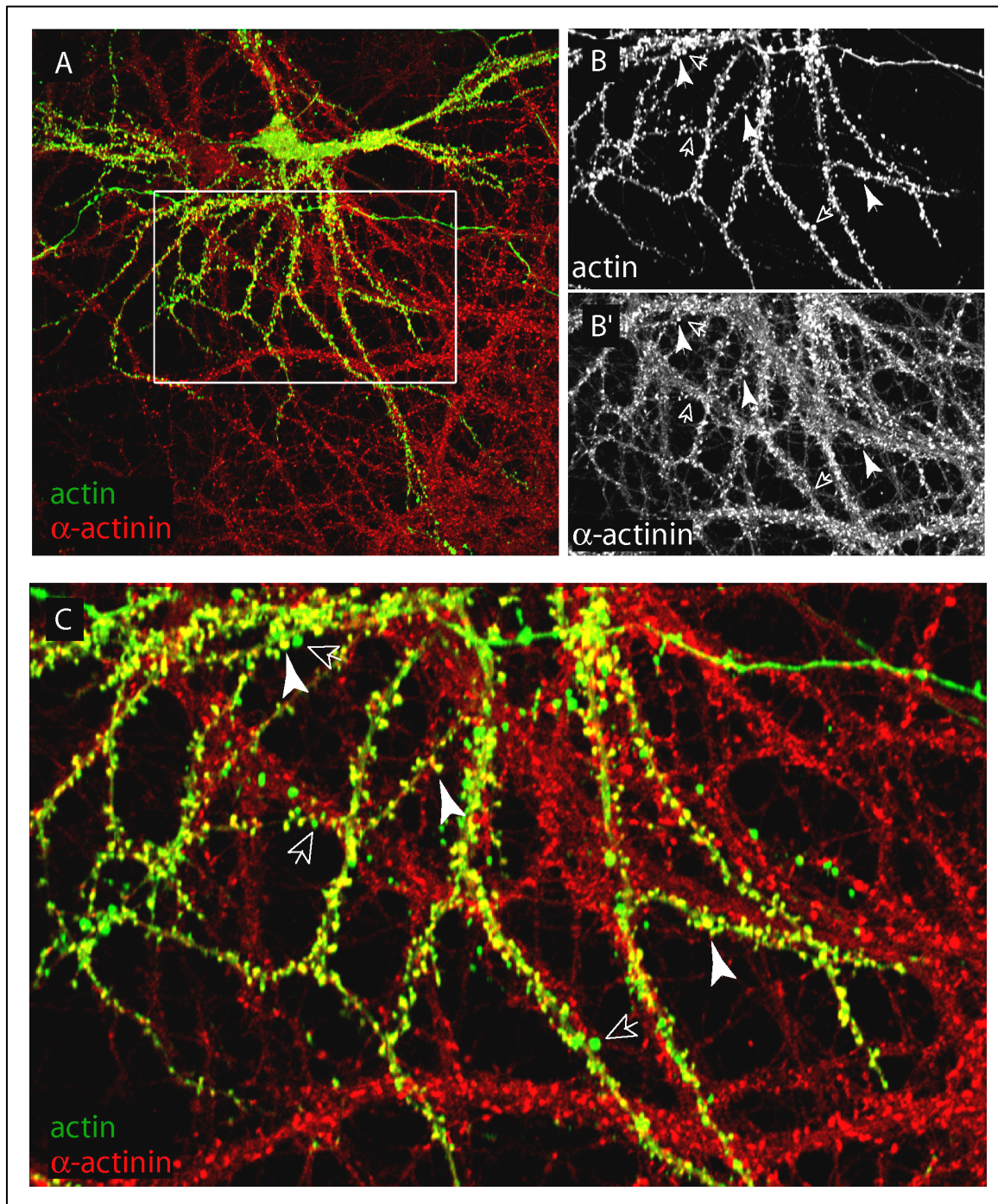


Figure 4: The crosslinking protein a-actinin is associated with a subset of dendritic spines

(A) Confocal image from a mature neuron transfected with GFP-actin (green) and counterstained with an antibody directed against a-actinin (red). a-actinin is expressed in mature neurons and also displays punctuate staining pattern. (B-C) Magnified images of area designated in A. (B) Dendritic segment designated in A, visualized by GFP-actin. (B') a-actinin visualized by immunoreactivity in area designated in A. a-actinin immunoreactivity is spotted around the dendritic shaft Closed arrowheads and open arrows). (C) Merge from B and B' (GFP-actin=green, a-actinin =red). Note that a-actinin is enriched in only a subset of spines along the designated dendrite (closed arrowheads). A significant number of spines do not show a-actinin staining (open arrows).

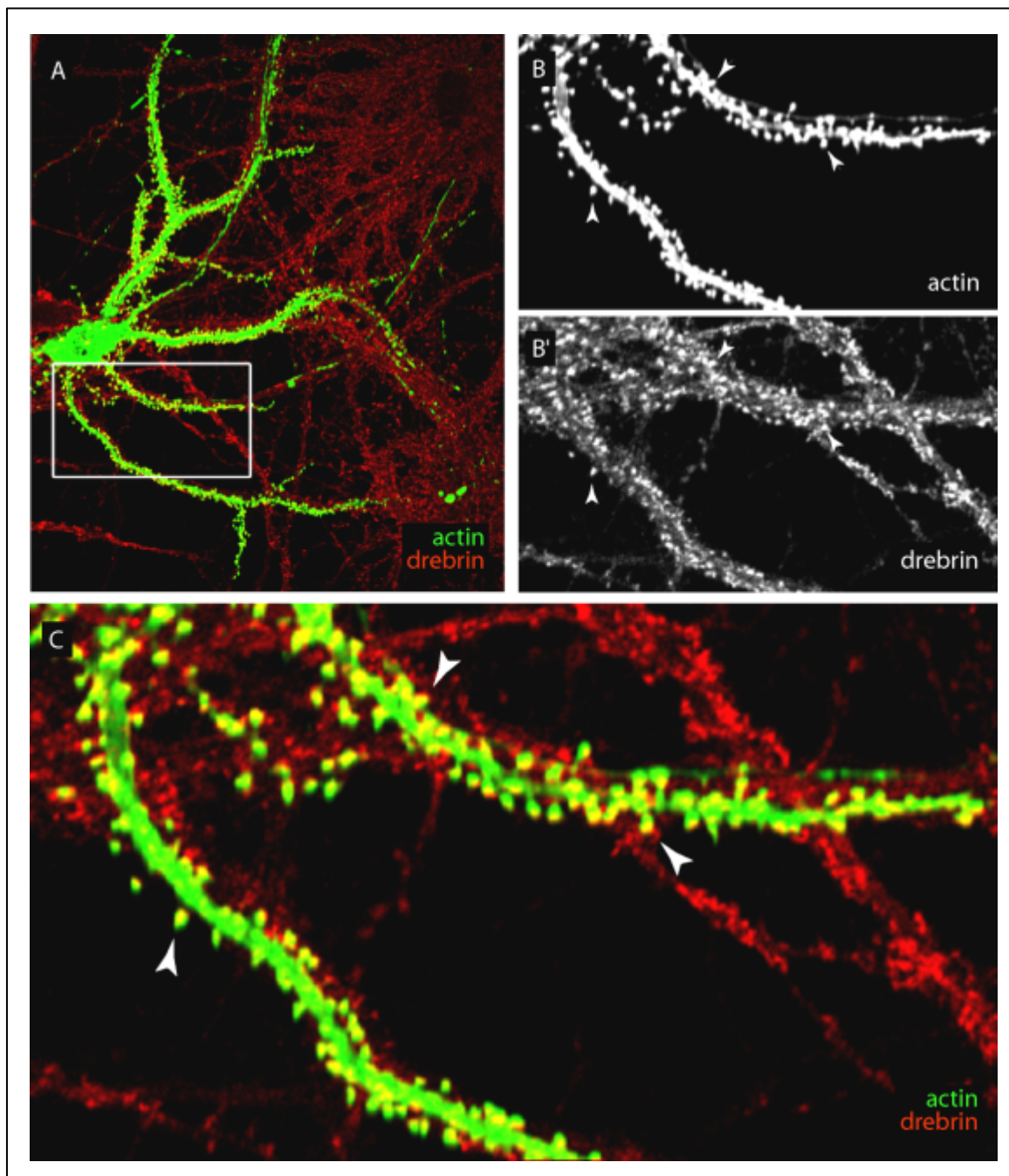


Figure 5: Drebrin is expressed in all dendritic spines

(A) Confocal image from a mature neuron transfected with GFP-actin (green) and counterstained with an antibody directed against drebrin (red). Drebrin is expressed in mature neurons and displays punctuate staining pattern. (B-C) Magnified images of area designated in A. (B) Dendritic segment designated in A, visualized by GFP-actin. (B') Drebrin visualized by immunoreactivity in area designated in A. Drebrin immunoreactivity appears as spots surrounding the dendritic shaft (closed arrowhead). (C) Merge from B and B' (GFP-actin=green, drebrin=red). Note that the drebrin is enriched in every single spine along the designated dendrite (closed arrowheads)

Distribution of proteins implicated in the anchorage of the actin cytoskeleton to the extracellular space or to neighboring cells in dendritic spines:

The cortical layers of most tissue cells are attached to the extracellular space or to neighboring cells via specialized regions in which bundles of actin are attached to the plasma membrane through specific sets of associated proteins ; the adherens junctions in cell-cell contacts (Gumbiner, 1996) and the focal contacts or adhesion plaques in cell substratum contacts (Parsons et al., 2000).

The cell receptors involved in the formation of focal contacts, the site in a cell where actin filaments are anchored to the extracellular matrix, belong to the large family of integrins (Burridge and Chrzanowska-Wodnicka, 1996), which span the plasma membrane in the region of focal contacts. Its cytoplasmic domain interacts with talin which in turns binds to vinculin. Vinculin interacts with α -actinin which in turns binds to actin filaments (Miyamoto et al., 1995).

Adherens junctions are the site on cortical actin where cell are attached to each other (Gumbiner, 1996). The apposing faces of an adherens junction are held together by membrane proteins capable of self association, principally the family of Ca^{2+} cells adhesion molecules, the cadherins (Petzelbauer et al., 2000). These span the plasma membrane having a cytoplasmic domain that becomes associated to the cytoskeleton via adaptor proteins, the catenins (Petzelbauer et al., 2000) and become associated to the actin cytoskeleton via the same linker proteins as focal contacts, vinculin (Weiss et al., 1998) and α -actinin (Knudsen et al., 1995).

We first looked at the distribution of β -catenin, an intracellular member of the cadherins complex to visualize adherens junctions. As seen in Figure 6A β -catenin is expressed in mature dispersed hippocampal neurons and displays a patchy distributions pattern. β -catenin staining was low in dendritic shafts and high in spots surrounding the dendritic shaft. These spots were closely associated with dendritic spines as can be readily seen at higher magnification (Figure 6B, B' and C).

Next, we looked at the universal linker between adhesion structures and the actin cytoskeleton, vinculin, showed a very different staining pattern. Vinculin is also

highly expressed in dispersed hippocampal neurons (Figure 6A), however, in contrast to β -catenin, it was diffusely distributed within the entire neuron and was not associated to any particular structure (Figure 6 B, B' and C).

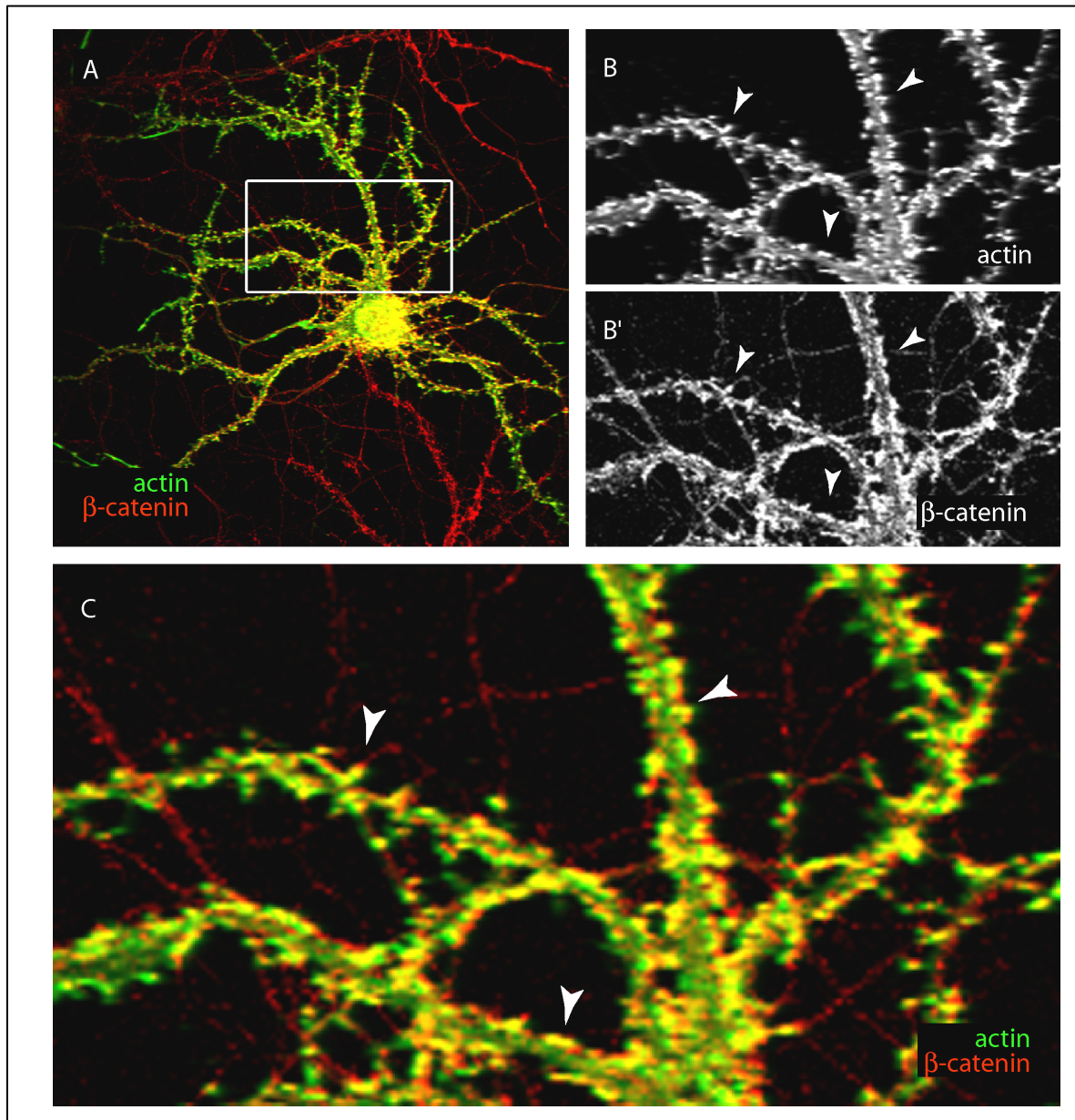


Figure6: b-catenin is localized at the synapse

(A) Confocal image taken from a mature neuron transfected with GFP-actin (green) and counterstained with an antibody directed against β -catenin (red). β -catenin is expressed in mature neurons where it is distributed in a patchy pattern. (B-C) Magnified images of area designated in A. (B) Dendritic segment designated in A, visualized by GFP-actin. (B') β catenin immunoreactivity in area designated in A. β catenin signal is high in dendritic protrusions and low in dendritic shaft. (C) Merge from B and B' (GFP-actin=green, β -catenin =red). Note that β -catenin is enriched in every single spine along the designated dendrite (closed arrowheads)

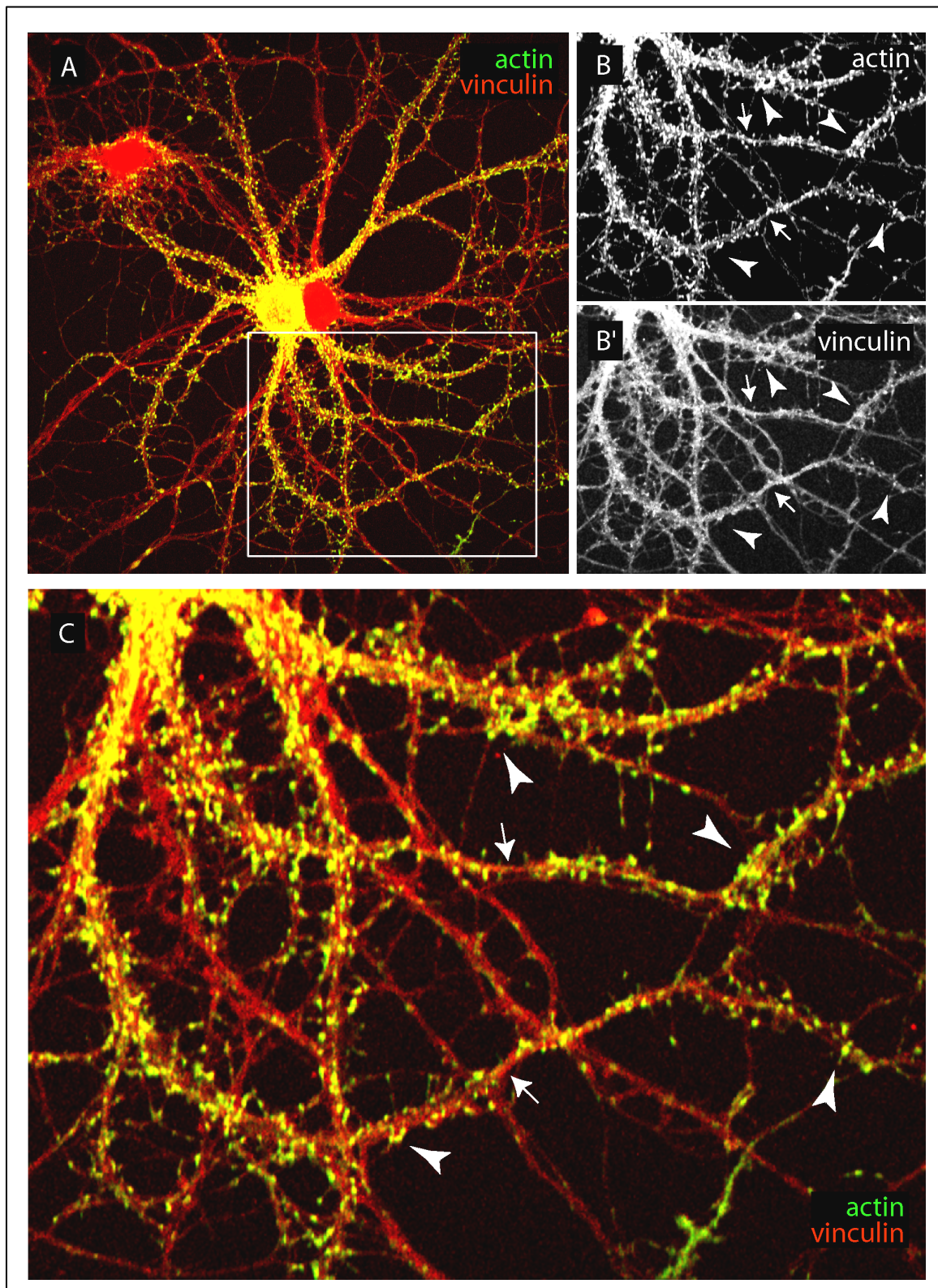


Figure7: Vinculin is diffusely distributed throughout dispersed hippocampal neurons

(A) Confocal image taken from a mature neuron transfected with GFP-actin (green) and counterstained with an antibody directed against vinculin (red). Vinculin is expressed in mature neurons and displays a diffuse staining pattern. (B-C) Magnified images of area designated in A. (B) Dendritic segment designated in A, visualized by GFP-actin. (B') Vinculin immunoreactivity in area designated in A. Vinculin is diffusely distributed within the dendrite and slightly enriched in dendritic protrusions (closed arrowheads). (C) Merge from B and B' (GFP-actin=green, vinculin=red). Note that every single spine along the designated dendrite exhibits vinculin signal (closed arrowheads).

DISCUSSION

In all cells the basis for shape and stability can be traced down to a network of filamentous structures known as the cytoskeleton. Dendritic spines are specialized regions on the neuronal dendrite periphery, which harbor the majority of excitatory synapses in the brain. There are three major classes of spines; stubby spines, thin spines and mushroom type spines (Peters and Kaiserman-Abramof, 1970). Spine shapes are thought to modify synaptic currents in a variety of ways, for example by amplifying potential at the synapse or by compartmentalizing calcium in spine heads (Bliss and Collingridge, 1993; Hausser and Roth, 1997; Svoboda et al., 1996). In addition, spines show a default motility (Fischer et al., 1998; Star et al., 2002) and have been reported to undergo substantial structural changes over hours and months (Grutzendler et al., 2002; Lendvai et al., 2000; Trachtenberg et al., 2002). The underlying cytoskeleton of dendritic spines is actin (Matus et al., 1982). Intermediate filaments and microtubules the two other constituents of the cytoskeleton are virtually absent from spines (Kaeck et al., 2001). Thus the actin cytoskeleton in spines must control morphology and motility as well as the attachment and recycling of receptors and scaffolding proteins.

The actin cytoskeleton can perform various functions, ranging from organelle trafficking to cell anchorage to the extracellular matrix and these functions have been shown to be orchestrated by a myriad of actin-binding proteins in non-neuronal-cells. However, to date little is known about the molecular composition of the actin cytoskeleton in dendritic spines.

So, what determines various spine morphologies or the differential stability or motility of individual spines? Gaining information about the molecular organization of actin cytoskeleton in dendritic spines might help us understanding the mode of action of how their morphologies and motility is regulated.

The present study demonstrates for the first time that three proteins previously reported to be involved in or even to be essential for rapid actin treadmilling at the leading edge of migrating cells (Pollard and Borisy, 2003) , the Arp2/3 complex, cofilin and gelsolin, are also enriched in dendritic spines. Indirect evidence suggests that the inherent motility of dendritic spines relies on fast actin treadmilling rather

than actomyosin motors since it is readily blocked by cytochalasin D, a drug that blocks actin polymerization by irreversibly capping actin filaments end, but not by application of drugs inhibiting actomyosin based contractions (Fischer et al., 1998).

The finding that the Arp2/3 complex, cofilin and gelsolin, molecules related to actin treadmilling (Amann and Pollard, 2001; Pollard et al., 2002), are major constituent of the actin cytoskeleton in dendritic spines further supports this notion and points toward a lamellipodia-like organization of actin in the motile corona surrounding spine heads.

However not all dendritic spines had detectable amounts of Arp2/3. Given the eminent importance of the Arp2/3 complex for actin treadmilling, this observation tempts to speculate that the relative amount of Arp2/3 within a spine might reflect its level of motility. Since glutamate receptor activation and subsequent influx of Ca^{2+} into the spine head blocks rapid spine motility (Fischer et al., 2000), it would be interesting to examine in the future whether synaptic activation or blockade of glutamate receptors triggers Arp2/3 relocalization in hippocampal neurons.

Cell morphology is typically regulated by both microtubules and F-actin networks. Since microtubules are omitted from dendritic spines, the assignment to shape this structure is left to the actin cytoskeleton. The actin structures which are characteristically responsible for cell shape are F-actin networks built by actin cross-linking or bundling proteins and by actin anchoring complexes such as focal adhesions, both structures being closely associated (Courtois et al., 1981; Fowler, 1986; Grazi et al., 1992; Schook et al., 1979).

Two proteins regulating supramolecular structures, α -actinin (Allison et al., 2000; Wyszynski et al., 1998) and drebrin (Hayashi et al., 1996), have already been reported to be enriched in dendritic spines and we confirmed these findings.

However, α -actinin was only present at detectable levels in ca. 90% of spines and relative α -actinin content could not be attributed to a specific spine structure, arguing against a fundamental role of α -actinin in ascertaining spine architecture. Nevertheless, α -actinin could still be implicated in modulating spine stability. Spine containing α -actinin could be more stable than ones without α -actinin. Nevertheless,

stable actin filaments and the regulation of these structures appear to be eminent to determine spine since drebrin was highly concentrated in all dendritic spines.

Finally we looked at the distribution of protein involved in anchoring the actin cytoskeleton to other cells or to the extracellular matrix. Vinculin immunoreactivity was ubiquitously distributed within mature and was not organized in distinct areas in dissociated neurons indicating strong adherens of those cells. Most adherens is probably associated to integrin-mediated adhesion to the extracellular matrix, as we find that, agreements with previous reports, the cell-cell adhesion molecule β -catenin is highly restricted to synaptic sites (Goda, 2002).

Part II:**A NOVEL DREBRIN-BASED MECHANISM REGULATING THE
STRUCTURE AND STABILITY OF DENDRITIC SPINES**

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SUMMARY

Within the brain the vast majority of excitatory synapses are made onto dendritic spines, small protrusions from the surfaces of dendrite with a characteristic shape consisting of a narrow neck and an expanded head. This morphological arrangement enables spines to act as individual biochemical compartments that regulate postsynaptic ion fluxes and transmission-dependent cellular responses, properties that are crucial for determining the integration of signals in brain circuits. Spines are also major sites of morphological plasticity, showing activity-dependent changes in size, shape and number that are coupled to alterations in characteristics of synaptic transmission such as the number and distribution of neurotransmitter receptors. This morphological plasticity depends on high concentrations of dynamic actin filaments in the spine cytoplasm which produce rapid, lamellipodia-like changes in surface configuration that are superimposed on the more stable fundamental structure. Despite the importance of this arrangement for determining synaptic function and plasticity very little is known about the mechanisms that determine how the balance between surface motility and fundamental stability is managed.

We show here that overexpression of drebrin, an actin-binding protein present in dendritic spines, leads to the collapse of actin networks in the spine cytoplasm and the selective destabilization of spine morphology without affecting other aspects of supramolecular structure in spines such as the clustering of postsynaptic receptors or contacts with presynaptic terminals.

INTRODUCTION:

Dendritic spines are specialized dendritic protrusions which act as integrating units of excitatory input for the majority of neurons in the brain. Their shapes differ over a large variety of morphologies ranging from short to long, thin to thick or headless to bearing large head structures (Chicurel and Harris, 1992). Despite being extensively studied, the function of dendritic spines is still not well understood but they are thought to influence synaptic transmission in various ways. First, dendritic spines increase the density of synapses and the interconnectivity between neurons. Second, they are thought to operate as electrical compartments with the neck constriction amplifying membrane potential at the synapse (Svoboda et al., 1996). Finally, they act as compartments restricting the diffusion of activated enzymes and second messenger such as calcium (Yuste et al., 2000).

In all these schemes, spine size and shape are critical variables to define locally the strength of a given input on the synapse. Additionally, it has become increasingly evident, that dendritic spines at established synapses are not fixed structures but on the contrary retain a large potential for morphological plasticity. Indeed dendritic spines display motility by default and both rapid variations in spines occurring within seconds and slower changes in the appearance and disappearance of individual spines over hours and days have been documented (Fischer et al., 1998; Kaeck et al., 1999; Trachtenberg et al., 2002). In particular, changes in dendritic spine density and shape occur in response to sensory deprivation as well as behavioral and experimental paradigms such as LTP, pointing towards a possible connection between modification of dendrites or dendritic spines profiles and experience dependent adaptation of the brain (Engert and Bonhoeffer, 1999; Geinisman et al., 2001; O'Malley et al., 2000; Toni et al., 1999). At present, the molecular mechanisms underlying these morphological changes are poorly understood but a common feature for a variety of forms of dendritic plasticity is the involvement of actin, which is highly enriched in dendritic spines (Kaeck et al., 1997; Matus et al., 1982).

Actin can exist in two basic configurations in a mammalian cell; a highly dynamic one characterized by rapid actin treadmilling, typical of lamellipodia and microspikes

at the leading edges of migrating cells and the tips of growth cones, or as relatively stable arrays such as the actin cables or stress fibers associated with quiescent fibroblasts (reviewed in Ayscough, 1998). These two structural states are defined by different sets of actin binding proteins which control the length, stability and strength of individual actin filaments as well as the number and pattern of inter-filament attachments. Indirect evidence suggests that two distinct pools of actin also coexist in dendritic spines. On one hand dendritic spines display rapid motility which can be blocked by the actin depolymerizing drug cytochalasin D (Fischer et al., 1998). On the other hand, an internal core of actin has been proven to be extremely resistant to disruption by cytochalasin D and can be only disassembled after prolonged exposure to the actin sequestering drug latrunculin A (Allison et al., 2000) pointing towards a slow turnover of these core actin filaments.

One candidate molecule for regulating the stable, core actin pool of dendritic spines is drebrin A, an F-actin binding protein enriched in brain compared to other tissues that is particularly associated with dendritic spines (Hayashi et al., 1996). Drebrin exists in two major isoforms generated from the same gene via alternative splicing; drebrin E which is highly expressed in all tissues during embryogenesis including brain and which remains ubiquitously expressed in all tissues except muscles in adulthood (Peitsch et al., 1999), and drebrin A which differs from drebrin E by the insertion of a 84 amino acid intron. Drebrin A is only expressed in neurons of the adult brain where it is exclusively localized to dendritic spines (Hayashi et al., 1996). Drebrin function is poorly understood: it has no actin severing, cross-linking or nucleating activity but binds to F-actin with high affinity *in vitro*. Furthermore, drebrin competes with the F-actin stabilizing proteins tropomyosin, α -actinin (Ishikawa et al., 1994) and fascin (Sasaki et al., 1996) in *in vitro* actin polymerization assays, suggesting that drebrin is involved in regulating the proportions of stable actin in the cell.

Previous studies have claimed that overexpression of drebrin in cultured neurons induces lengthening of dendritic spine necks (Hayashi and Shirao, 1999), suggesting a role for this protein in determining spine morphology. However, so far the influence of drebrin on actin-based dendrite motility had not been evaluated. Because of the fundamental role of actin dynamics in spine plasticity both during development and

after synapses are formed, we have examined the role of drebrin by expressing green fluorescent protein (GFP) tagged drebrin in cultured neurons. Our results show that drebrin expression is linked to mobilization of the actin cytoskeleton. Drebrin overexpression resulted in the growth of filopodia and highly motile polymorphic spines from the dendrites of mature neurons. This effect was also observed when drebrin overexpression was induced in neurons after maturation had occurred. In our experiments, the unique F-actin binding site of drebrin (Hayashi et al., 1999) was sufficient to trigger the effect, indicating that the mode of action of drebrin is to compete with other F-actin binding proteins. Indeed, remobilization of spines was accompanied by the loss of immunostaining for the actin cross-linking protein α -actinin in dendritic spines of transfected drebrin-overexpressing neurons.

Taken together, these results suggest that drebrin plays a key role in regulating the morphological stability of synapses by controlling the ratio of stable, cross-linked actin filaments in mature neurons, which appears to be crucial for spine stability.

RESULTS

Drebrin over-expression destabilizes dendritic spine morphology:

To study the influence of drebrin on spine morphology and motility we expressed the adult brain isoform of drebrin (drebrin A) tagged with GFP in cultured hippocampal neurons and recorded the fluorescence by live cell imaging. Other cultures were transfected with GFP-actin to monitor the state of the actin cytoskeleton under control conditions (Fischer et al., 1998). Viewed by GFP-actin fluorescence, the dendrites of hippocampal neurons maintained in culture for three weeks or more have dendritic spines of mature appearance typically consisting of an expanded head, where the synaptic contact is made, connected to the dendritic shaft by a narrow neck (Figure 1A₁). In transfected neurons overexpressing drebrin-GFP, this mature spine morphology was destabilized so that dendrites produced a mixture of normal and polymorphic spines as well as filopodia lacking bulbous heads (Figure 1A₂). Because these effects may have resulted from overexpressed drebrin interfering with molecular mechanisms of spine maturation, we performed experiments where neurons were transfected after 18 days in culture by which time spine maturation is well advanced (Bartlett and Banker, 1984). The subsequent overexpression of drebrin-GFP at >20 DIV (Figure 1A₃) produced the same aberrant filopodial and polymorphic dendritic protrusions as when drebrin was overexpressed throughout development, thus indicating that drebrin overexpression can destabilize the structure of mature spines.

To verify that these effects were not artifacts caused by expressing the drebrin-GFP fusion protein, we repeated these experiments using a bicistronic expression construct in which unmodified drebrin was expressed in parallel with GFP-actin. Images of GFP-actin in drebrin-overexpressing dendrites (Figure 1A₄) revealed filopodia and polymorphic spines indistinguishable from those produced by the drebrin A-GFP fusion construct indicating that the destabilization of spine structure is indeed the result of drebrin overexpression. Figure 1B shows the distribution of mature normal spines ('globular'), polymorphic spines and filopodial protrusions in control GFP-actin transfected cells and in the various drebrin-overexpressing cells described

above. In control GFP-actin cells filopodia and polymorphic spines were rare, constituting less than 20% of all dendritic protrusions. By contrast in all the drebrin overexpression experiments globular, polymorphic and filopodial protrusions were present at equivalently increased levels.

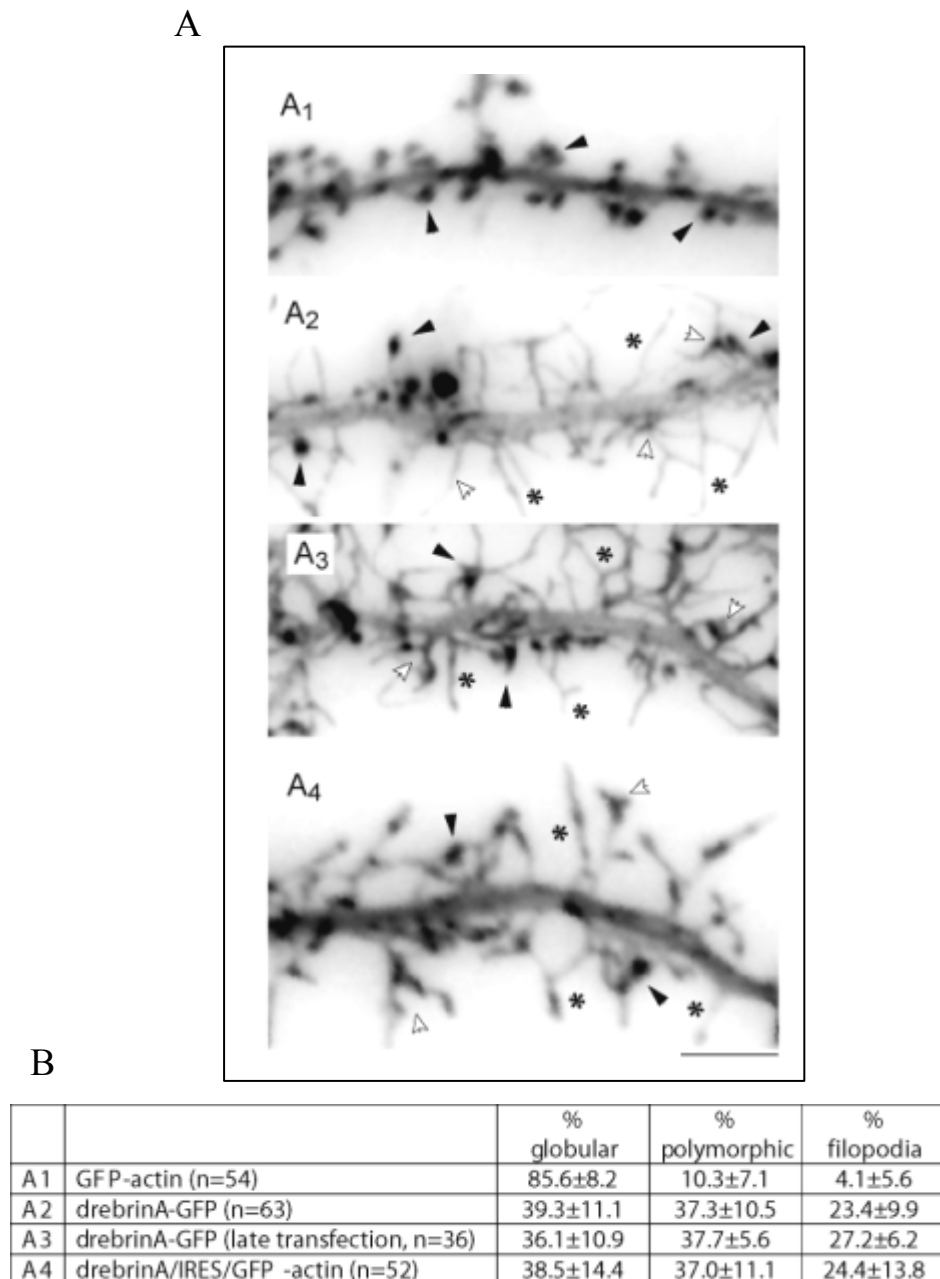


Figure 1: Drebrin A over-expression induces filopodial outgrowth from dendrites in hippocampal cultures: Dendritic segments taken from hippocampal neurons after 21 days in culture expressing (A₁) GFP-actin, (A₂) drebrin A-GFP, (A₃) drebrin A-GFP transfected after 18 DIV (late Transfection) or (A₄) drebrin A/IRES/GFP-actin. The contrast has been inverted to make fine protrusions more readily visible. (A₁) GFP-actin expressing neurons display bulb-shaped spines (filled arrowheads) and are devoid of filopodia. (A₂) In contrast drebrin A-GFP expressing neurons grow filopodial protrusions (asterisks) and polymorphic spines (open arrows) along with bulb-shaped spines (filled arrowheads) from their dendrites. (A₃) Over-expressing drebrin A-GFP after synapse maturation or (A₄) overexpressing an untagged version of drebrin A resulted in the same abnormal filopodial outgrowth (asterisks) and generation of polymorphic spines (open arrows). Scale bar=5 μ m. (B) Table showing percentage of filopodia, polymorphic spines or globular spines per randomly selected 50 μ m portion of dendrite on dendrites of GFP-actin, drebrin A-GFP, drebrin A-GFP late transfection or drebrin A/IRES/GFP-actin transfected neurons (mean \pm SEM; n=numbers of cells for which segments were analyzed).

Figure 2 illustrates the major varieties of aberrant spine like structures, excluding filopodia, in drebrin overexpressing cells. These include normal globular (Figure 2A₁) and polymorphic spines, which could be classified into the following categories; bifurcated (Figure 2A₂), bearing head filopodia (“thorny”) (Figure 2A₃) and irregular (Figure 2A₄) spines. These aberrant shapes were only rarely encountered in controlled GFP-actin expressing cells (Figure 2B), where dendritic spines of typically mature appearance accounted for ca. 90% of dendritic protrusions. The presence of both aberrant protrusions and dendritic spines of normal appearance on drebrin overexpressing cells suggests that the increased drebrin levels produced by transfection selectively destabilizes spine structure. Our results differ with previous reports from Hayashi and Shirao (Hayashi and Shirao, 1999) in that they show gross changes in spine morphologies induced by drebrin rather than merely the lengthening of spine necks. This may reflect the fact that the apparent lengthening effect by drebrin is primarily a result of the loss of their compact structures.

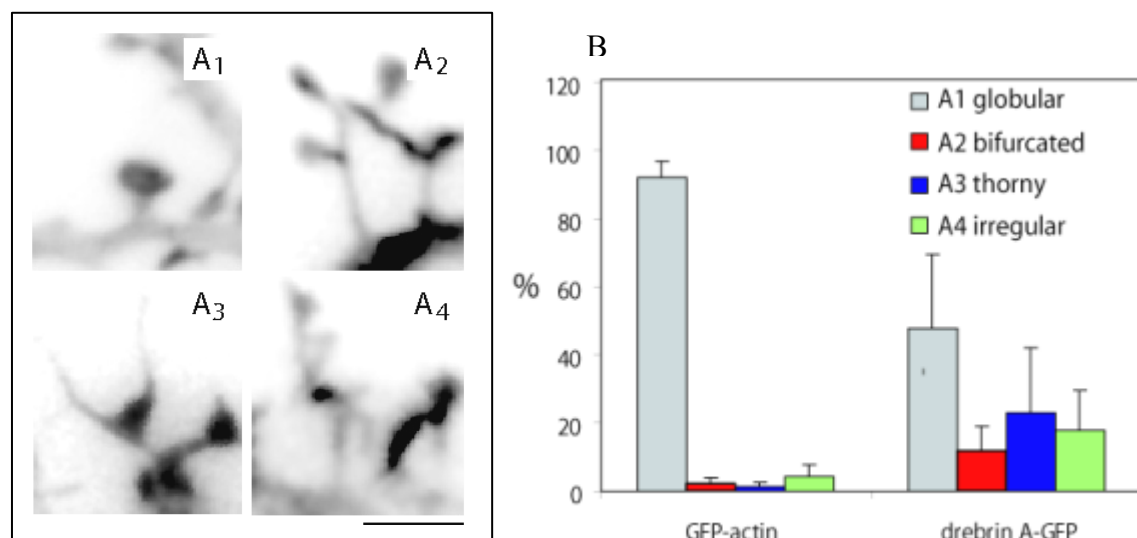


Figure 2: Drebrin overexpression produces several types of polymorphic spines along with normal globular spines in mature neurons:

Representative spines of (A₁) globular, (A₂) bifurcated, (A₃) thorny or (A₄) irregular shape. Scale bar 2 μm. (B) Mean percentage of globular, bifurcated, thorny or irregular spines per 50 μm segment of dendrite of GFP-actin versus drebrin A-GFP expressing neurons. Data from drebrin A-GFP (early and late transfection) and drebrin A/IRES/GFP-actin expressing neurons were pooled (GFP-actin: n= 54, drebrin A: n= 75, where n=numbers of cells for which segments were analyzed).

Although drebrin A-GFP expressed in these experiments was distributed throughout the neuronal cytoplasm, there was no change in the number of terminal dendrites in drebrin overexpressing cells compared to GFP-actin controls (drebrin A-GFP = 14.6 ± 8.7 dendrites/cell, $n=52$; GFP-actin = 15.9 ± 6.7 dendrites/cell, $n=43$) indicating that dendritic arborization was unaltered. Overall dendrite length was also unaffected by drebrin A over-expression (GFP-actin = 18.38 ± 7.29 μm , drebrin A-GFP = 18.07 ± 8.06 μm).

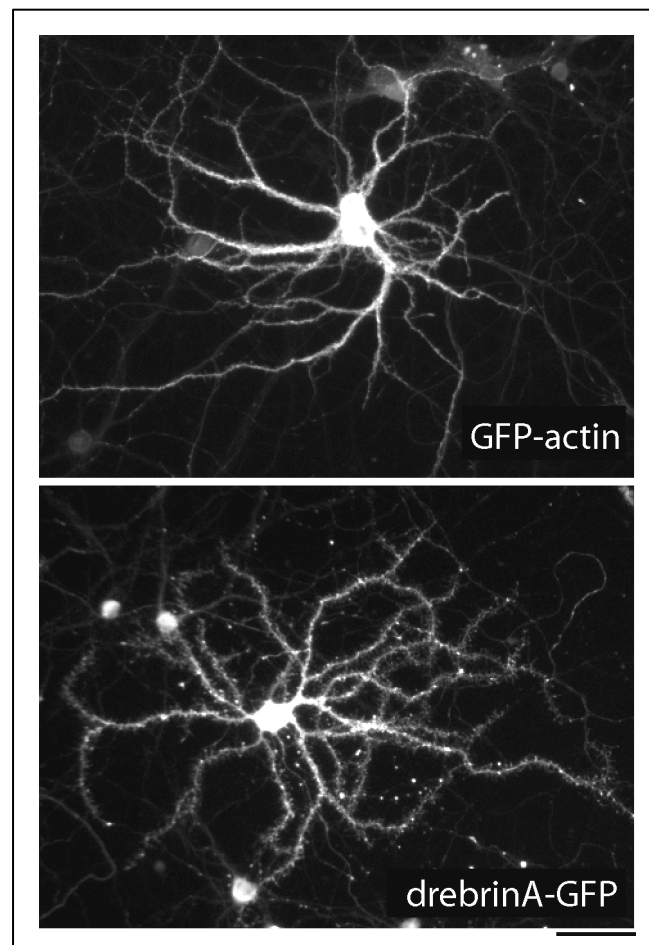


Figure 3: Drebrin has no effect on dendrite arborization or length:

Representative hippocampal neurons transfected with either GFP-actin or drebrinA-GFP and maintained in culture for 21 days. Both types of neurons build up an elaborate dendritic tree which show similar branching pattern and grow dendrites of comparable length.

Drebrin-induced aberrant protrusions are highly motile:

It has been previously shown that dendritic spines display rapid inherent motility. In agreement with those previous reports, time-lapse recordings from mature control GFP-actin expressing neurons showed that motility was (Fischer et al., 1998) restricted to lamellipodia-like ruffling at the ends of spine heads (Figure 4A₁, 4A₁' and supplementary movie 1). By contrast parallel experiments on drebrin A-GFP overexpressing neurons showed that drebrin-induced aberrant protrusions were extensively motile undergoing rapid changes in overall shape and frequently producing long filopodia rapidly extending and retracting from spine heads (Figure 4A₂, 4A₂' and supplementary movie 2).

Figure 4B₁ and 4B₂ illustrate the enhanced length and motile behavior of motile protrusions on globular or polymorphic spines from control versus drebrin A overexpressing neurons, by plotting the total length (*l*) of four protrusions extending from dendritic spines head of GFP-actin (Figure 4B₁) or drebrin A-GFP (Figure 4B₂) transfected neurons.

We next addressed the question of the relationship between drebrin and actin in drebrin destabilized dendritic protrusion. Figure 5 shows a segment of a dendrite from a hippocampal neuron simultaneously transfected with CFP-actin and drebrin-YFP to allow their relative distributions to be observed as shape changes occurred. As seen in the merged red/green image in the lower row, in all categories of protrusion, including thin filopodia emerging from spine heads, CFP-actin and drebrin-YFP were colocalized, implying that they interact in these destabilized structures. We frequently encountered aberrant processes within which drebrin-actin aggregates changed position during time-lapse recording (for example Figure 5, asterisk, see also supplementary video data to Figure5).

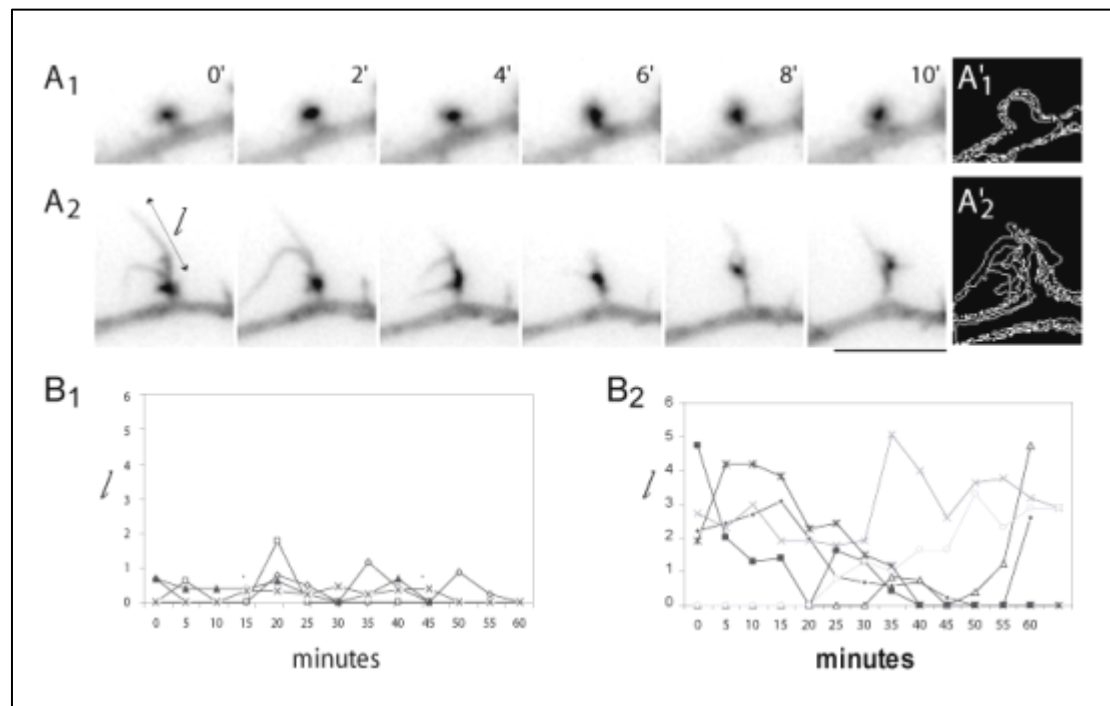


Figure 4: Drebrin induces highly motile protrusions on mature dendrites:

Single frames taken from a 10 minutes time-lapse recording of a (A₁) mature GFP-actin expressing neuron and (A₂) a drebrin A-GFP expressing neuron. (A'₁ and A'₂) Overlays of thresholded outlines from six successive time intervals, two minutes apart, from the spines designated in A₁ or A₂. Drebrin A-GFP transfected neurons display rapid extension of spine head filopodia as well as irregular highly plastic spine heads while spines on mature control neuron retain their globular shape. Scale bar = 5 μ m.

(B₁ and B₂) Graphs showing absolute length (l) of four different filopodia on four different spine heads of (B₁) GFP-actin expressing or (B₂) drebrin A-GFP over-expressing neurons in μ m plotted versus time.

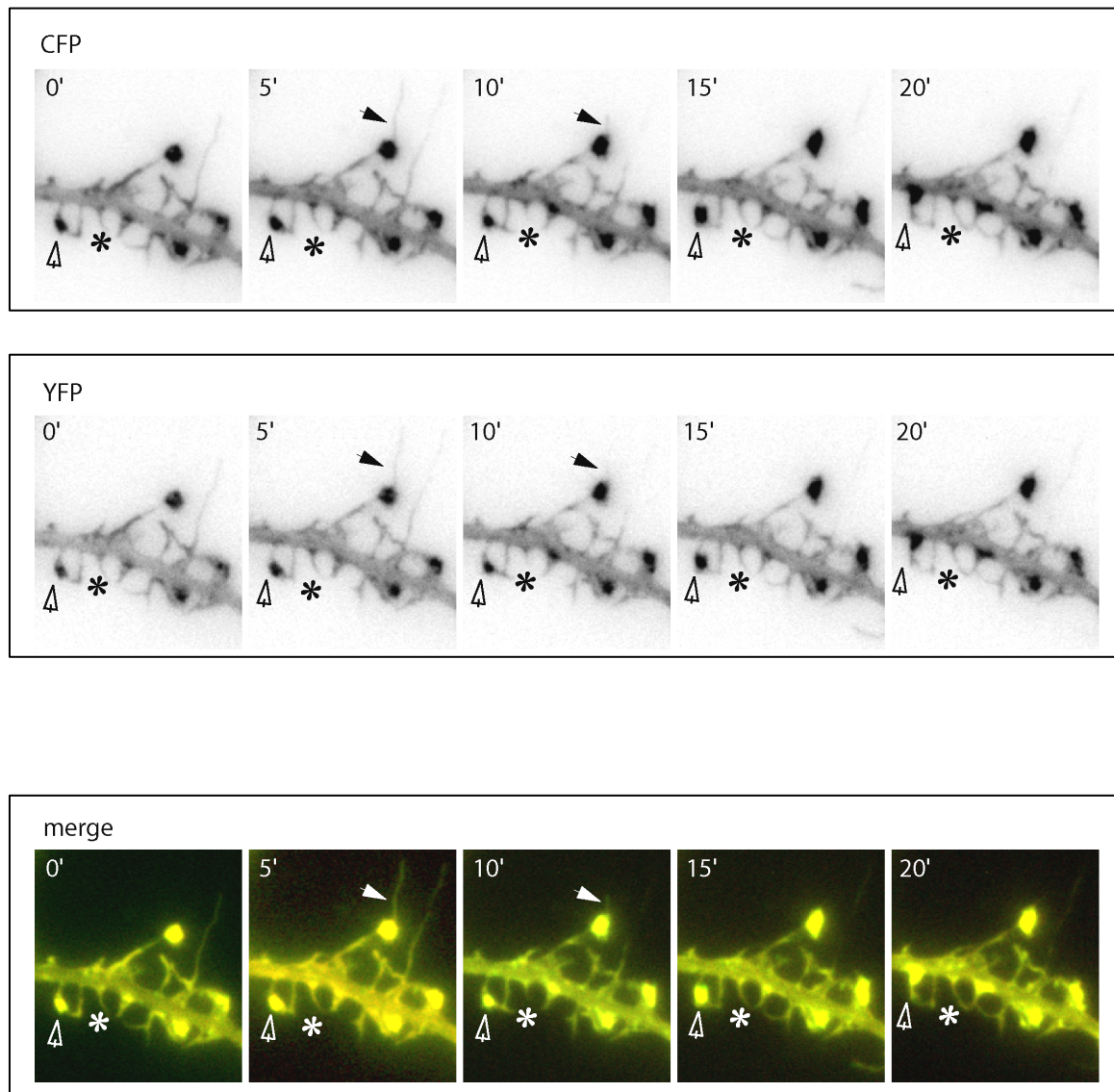


Figure 5: Overexpressed drebrin is closely associated with actin:

Single frames taken from timelapse recording of a dendrite from a neuron double transfected with CFP-actin and drebrin A-YFP. Upper panel: CFP-actin. Middle panel: drebrin A-YFP. Lower panel: merged image from red/green coded CFP-actin/drebrin A-YFP images. CFP-actin and drebrin A-YFP signals overlap completely in filopodia (asterisk), head filopodia (closed arrows) and drebrin/actin clusters that move along protrusions (open arrows) demonstrating the close association of the two proteins. Scale bar = 5 μ m.

To ensure that the rapid morphological changes described above were due to genuine motility and not focus shifts we recorded Z-series stacks of confocal images from drebrin A-GFP transfected neurons at 10 min intervals and confirmed that the dynamic behaviour of drebrin A induced protrusions was visible in all image planes (see supplementary movies).

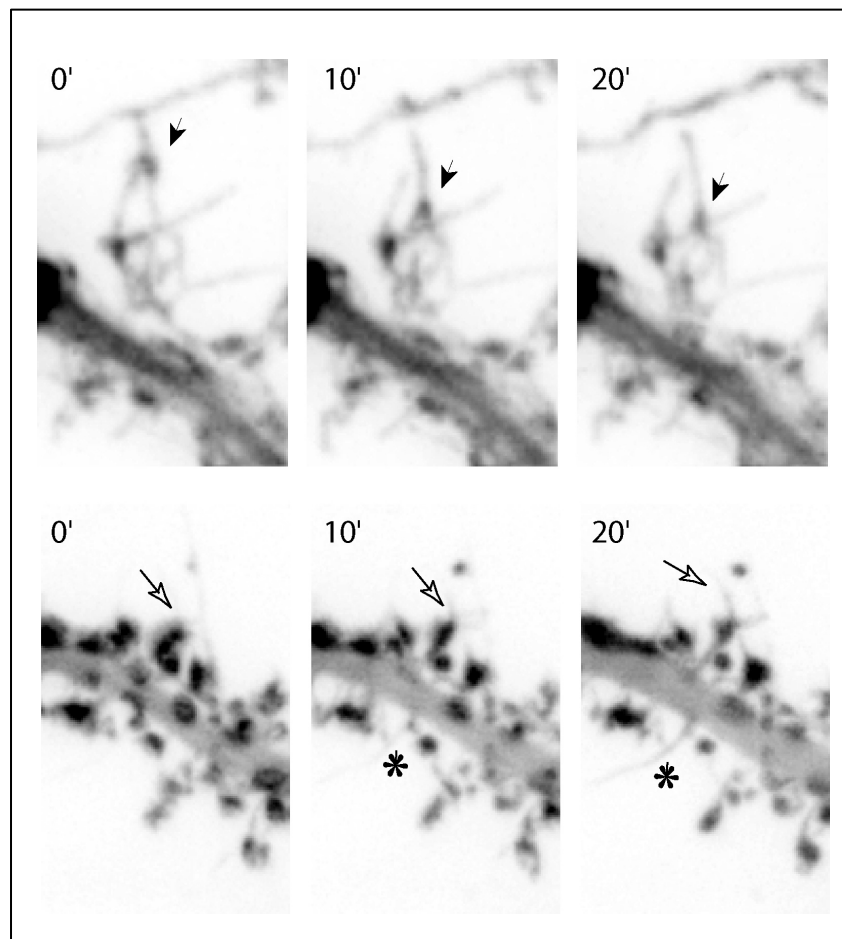


Figure 6:

Two dimensional projections of Z-stacks from dendrites of two different drebrin A-GFP transfected neurons, taken 10 minutes apart. Drebrin-induced polymorphic protrusions are highly motile showing drebrin clusters traveling along protrusions (closed arrow), motile filopodia growing from the dendritic shaft (asterisk) and from the head of polymorphic spines (open arrow).

Spatial distribution of synaptic structural components in drebrin-induced protrusions:

To determine whether protrusions on drebrin overexpressing dendrites are innervated we fixed drebrin-GFP transfected neurons after 21 DIV and counterstained them with antibodies against the presynaptic vesicle-associated protein synaptophysin to reveal presynaptic buttons. As shown in Figure 7, the state of innervation depended on the morphology of the postsynaptic structure. Both spines (closed arrows) and aberrant polymorphic spines (asterisks) were apposed to synaptophysin-positive puncta which contacted the spine head, whereas presynaptic contacts were never visible on headless filopodia (open arrowheads)

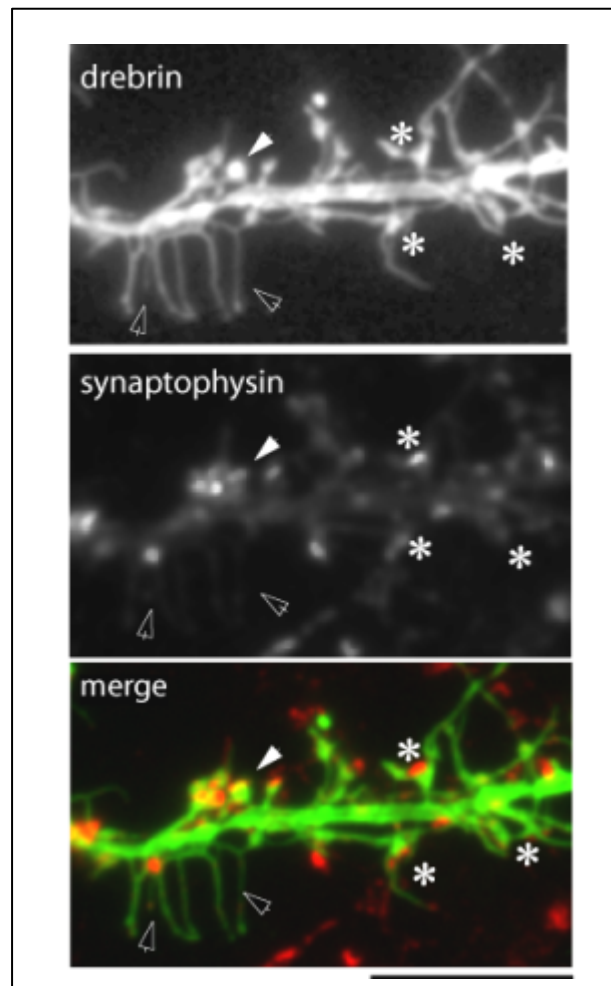


Figure 7: Drebrin-induced polymorphic spines are innervated:

Dendritic portion of a neuron transfected with drebrin A-GFP, fixed at 21DIV and stained with an anti-synaptophysin antibody. Filopodia are devoid of presynaptic terminals (open arrows), while both bulb-shaped (closed arrows) and polymorphic spines (asterisks) are innervated (merge: GFP=green synaptophysin=red). Scale bar = 10 μ m.

To further characterize drebrin-induced protrusions we used specific antibodies to determine the distributions of the postsynaptic scaffolding protein PSD-95 and the glutamate receptor subunits GluR1 and NR1. We found that all of these components are normally clustered within spine heads, consistent with previous immunohistochemical studies of control fixed neurons (Allison et al., 2000; Allison et al., 1998).

Figure 8 shows the distribution of these proteins in control GFP-actin transfected cells and drebrin expressing neurons. Within the heads of drebrin-induced polymorphic spines all three proteins formed focal clusters as they did in the morphologically normal, globular dendritic spines of GFP-actin expressing control cells (compare Figures. 8A, 8B and 8C to 8A', 8B' and 8C'). However, PSD95, GluR1 and NR1 never formed clusters in headless filopodia (Figure 8A', 8B' and 8C'). These results indicate that morphological differentiation of the postsynaptic site develops normally despite drebrin-induced destabilization of overall dendritic spine structure.

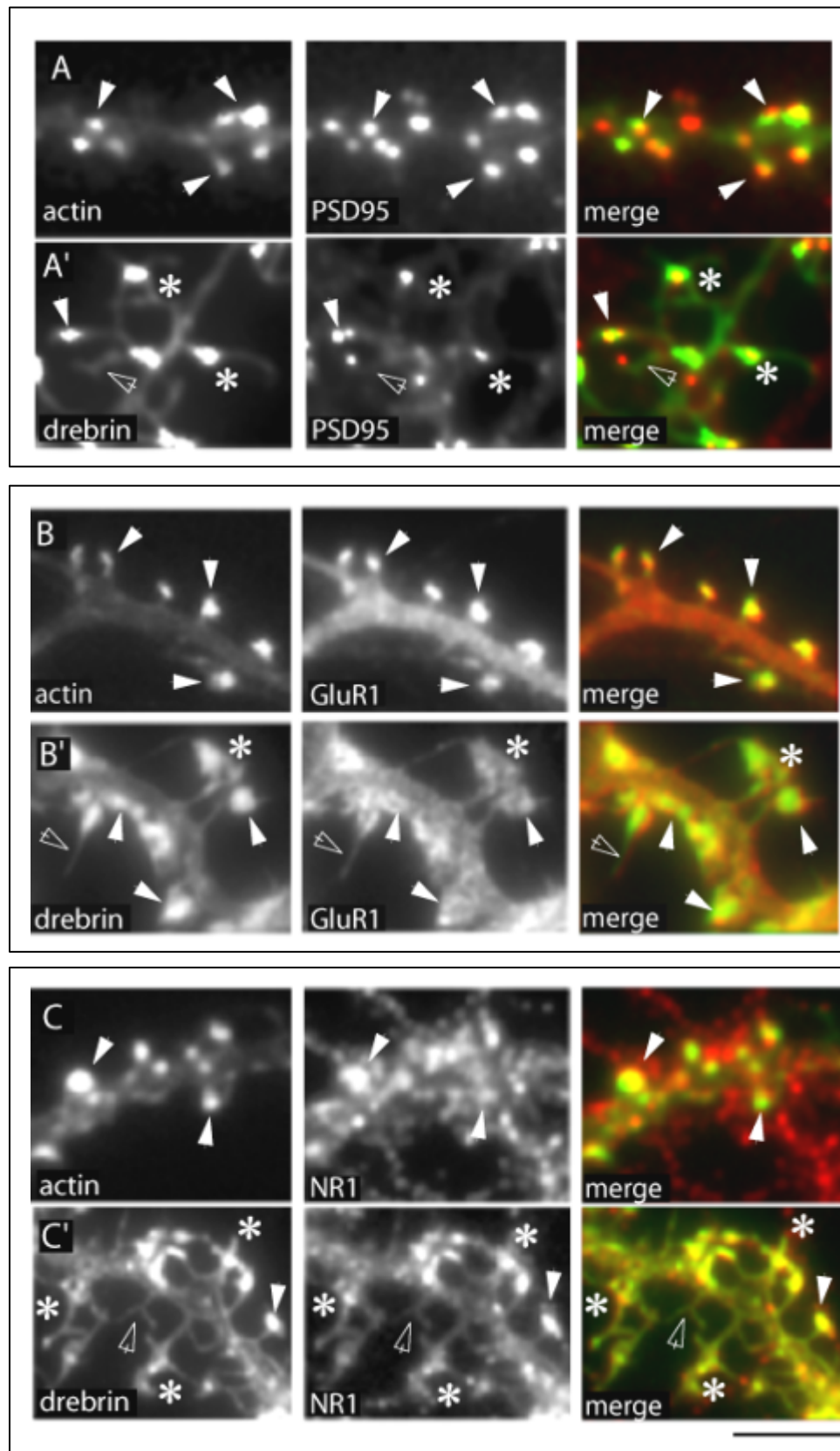


Figure 8: Drebrin-induced polymorphic spines contain postsynaptic molecule clusters:

(A-C') Hippocampal neurons were transfected with GFP-actin (A, B and C) or drebrin A/IRES/GFP-actin (A', B' and C'), fixed at 21DIV and stained with either (A and A') anti PSD-95, (B and B') anti GluR1 or (C and C') anti NR1 antibodies. Left panel: GFP-fluorescence, middle panel: PSD-95, GluR1 or NR1 immunoreactivity and right panel merge: GFP=green, PSD-95, GluR1 and NR1=red. Scale bar = 5 μ m.

Regulation of rapid actin based motility in drebrin-induced polymorphic protrusions:

One possible explanation for the destabilization of spine morphology by drebrin is that its over-expression interferes with the regulation of spine motility via postsynaptic glutamate receptors, whose activation normally suppresses actin dynamics and the associated changes in spine shape (Fischer et al., 2000; Star et al., 2002). To see whether this was the case we assessed actin dynamics in time lapse recordings from drebrin overexpressing neurons before, during and after exposure to AMPA.

Figure 9 shows that applying 2 μ m AMPA to dendrites rapidly suppressed changes in spine shape and induced an increase in a calculated shape factor indicative of the disappearance of dynamic protrusion from spine heads similar to that seen in dendritic spines of GFP-actin expressing neurons (Fischer et al., 2000). Thus regulation of spine motility via postsynaptic AMPA receptors remains intact on drebrin overexpressing neurons, suggesting that drebrin-induced destabilization of dendritic spine structure involves mechanisms distinct from those that regulate rapid actin dynamics in dendritic spines.

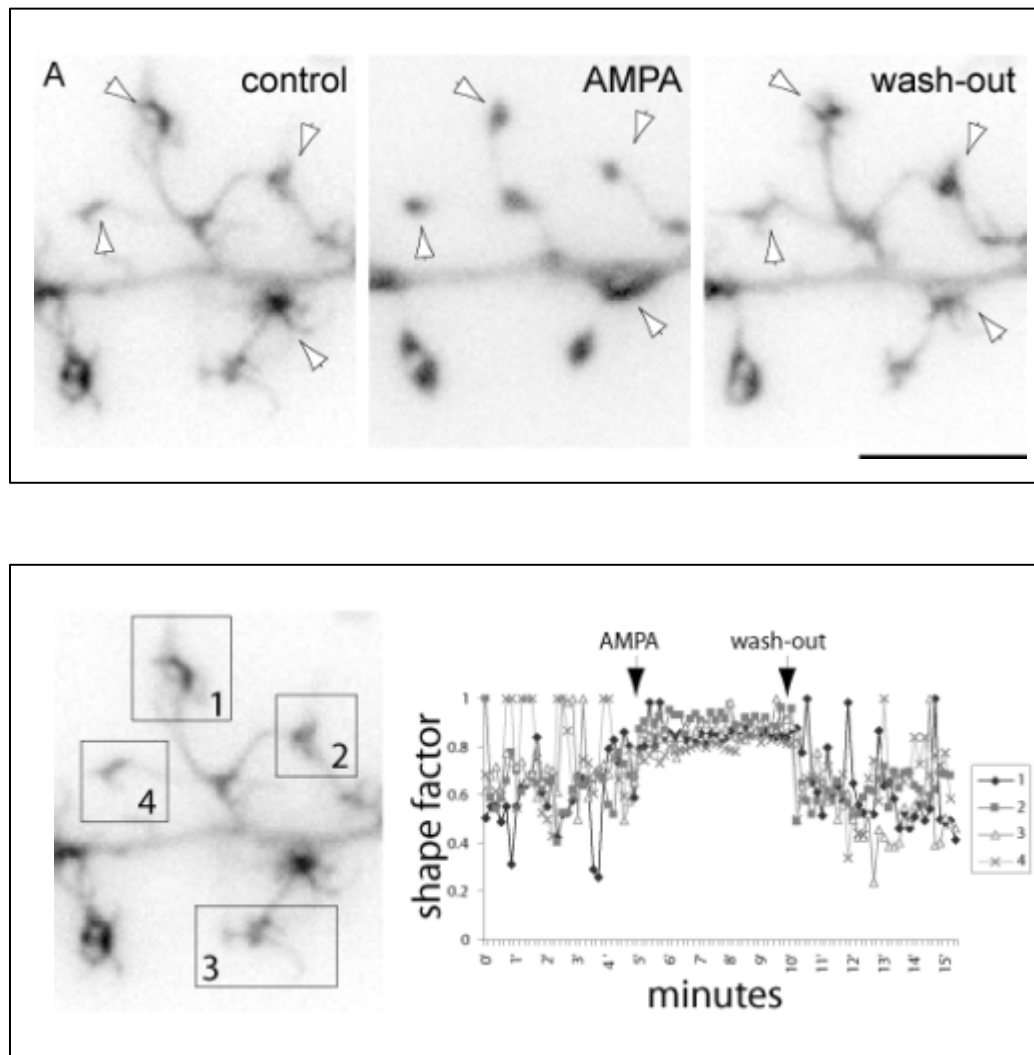


Figure 9: Glutamate-receptor activation blocks motility:

(A) Single frames from a 15 minutes time-lapse recording of a dendrite from a drebrin A-GFP transfected neuron where $2\mu\text{M}$ AMPA was applied after 5 minutes and washed away after additional 5 minutes. Polymorphic spines (arrows) show a reversible stop after AMPA treatment. (B') The Plot shows the shape factor (see materials and methods) of the four spines assigned in (B) during the 15 minutes recording. Scale bar = $5\mu\text{m}$.

The actin-binding domain of drebrin is sufficient to destabilize spine structure :

Drebrin is a multifunctional protein containing a variety of discrete functional domains. Additionally it occurs as two isoforms, generated from the same gene via alternative splicing, a ubiquitous form which is expressed in the embryonic brain (drebrin E) and an adult isoform, drebrin A, which contains an additional 84 amino acid insert and is expressed only in the adult brain (Shirao, 1995). To assess the roles of these isoforms and their component domains in the destabilization of spine structure we performed a transfection analysis using the constructs shown diagrammatically in Figure 10.

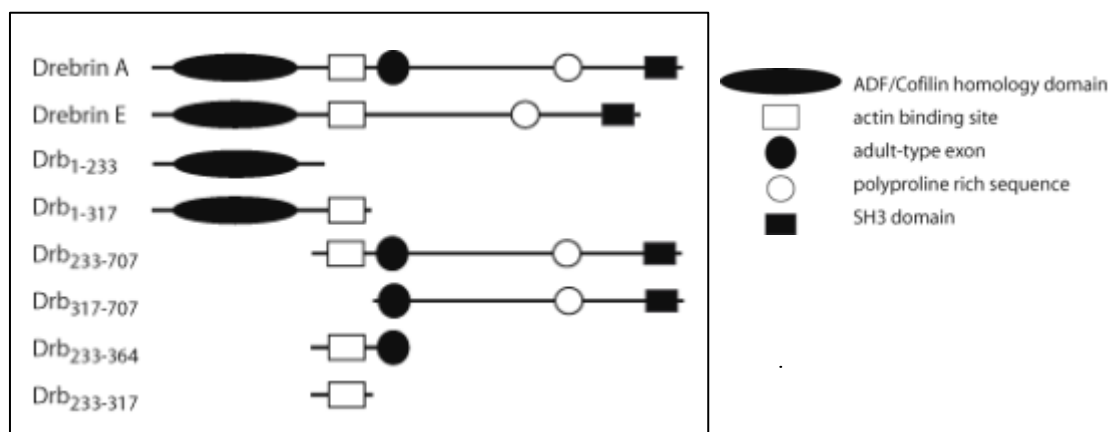


Figure 10: Schematic drawing of drebrin mutants used in this study. All mutants were used as both GFP- and hemagglutinin (HA)-tagged constructs.

Comparing adult drebrin A to embryonic drebrin E (Figure 11B and 11C) failed to reveal any difference in the production of polymorphic and filopodial protrusions. Further experiments with a range of deletion constructs showed that only constructs containing the drebrin actin-binding domain were effective in destabilizing spine structure. Moreover, constructs containing the actin binding domain performed indistinguishably from one another and were as effective as wildtype drebrin A or drebrin E. Additionally a construct consisting only of the drebrin actin binding domain was destabilized spine structure, producing polymorphic spines and filopodia as effectively as wildtype drebrin (Figure 11B and 11C). Thus the actin binding

domain of drebrin is both necessary and sufficient for triggering the destabilizing effect of drebrin on dendritic spine morphology.

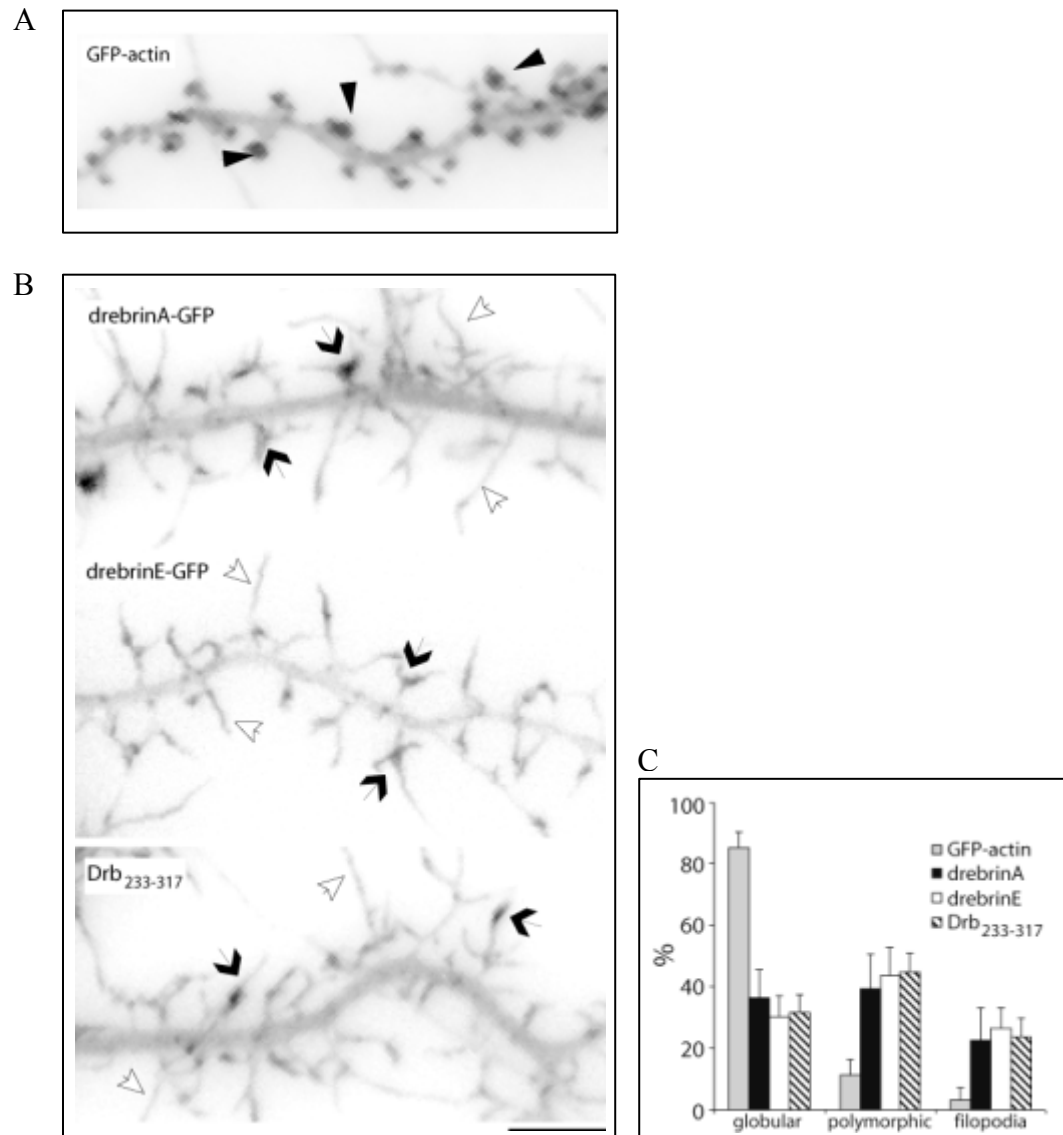


Figure 11: The actin binding site of drebrin is sufficient and necessary to produce filopodia and polymorphic spines.

Dendritic segments taken from neurons transfected with either (A) GFP-actin, (B) drebrin A-GFP, drebrin E-GFP or the actin binding domain of drebrin, Drb₂₃₃₋₃₁₇. (A) Dendrites on control neurons have bulb-shaped spines (black arrowheads) while (B) neurons transfected with embryonic drebrin E, adult drebrin A or the actin binding site of drebrin alone (Drb₂₃₃₋₃₁₇) showed filopodia (open arrowheads) and polymorphic spines (arrows). Scale bar = 5 μ m. (C) Quantitative analysis of protrusions on dendrites of GFP-actin, drebrin A-GFP, drebrin E-GFP or Drb₂₃₃₋₃₁₇ transfected neurons as mean percentage of class of protrusion accounted for as filopodia, polymorphic or globular spines per 50 μ m of dendrite.

Drebrin displaces α -actinin from dendritic spines:

We next considered the mechanism by which drebrin binding to actin may regulate dendritic spine stability. Drebrin has no known effect on actin turnover nor does it show any actin bundling, capping or severing activity (Ishikawa et al., 1994). However, previous experiments have shown that drebrin competes for binding to actin filaments with the actin-bundling proteins fascin (Sasaki et al., 1996), α -actinin and tropomyosin (Ishikawa et al., 1994). To establish whether competition between drebrin and actin-bundling proteins occurs in polymorphic dendritic protrusions of drebrin overexpressing cells, we examined the distribution of α -actinin (Figure 12) and two non-bundling actin binding proteins, p-34-arc, a component of the actin nucleating complex arp2/3 (Figure 13), and gelsolin (Figure 14) in drebrin-overexpressing cells.

In control GFP-actin transfected cells α -actinin was colocalized with actin in spine heads (Figure 12A and 12A', closed arrows). By contrast, in drebrin overexpressing cells, there was a striking absence of α -actinin from spines, which also exhibited the expected drebrin-induced aberrant morphology (Figure 12B and 12B', open arrowheads). Additionally, α -actinin levels were markedly decreased in the dendrites of drebrin overexpressing cells, indicating that drebrin influences α -actinin expression and localization in dendrites.

In contrary, both gelsolin and the Arp2/3 complex, which are concentrated in spine heads of control GFP-actin transfected cell (Figure 13A, A' and 14 A, A'; closed arrows), remain associated with spines and are expressed at unchanged levels in dendrites and dendritic protrusions of drebrin overexpressing neurons (Figure 13 B, B' and 14B, B'; closed arrows). Together these results indicate that the level of drebrin in dendrites influences.

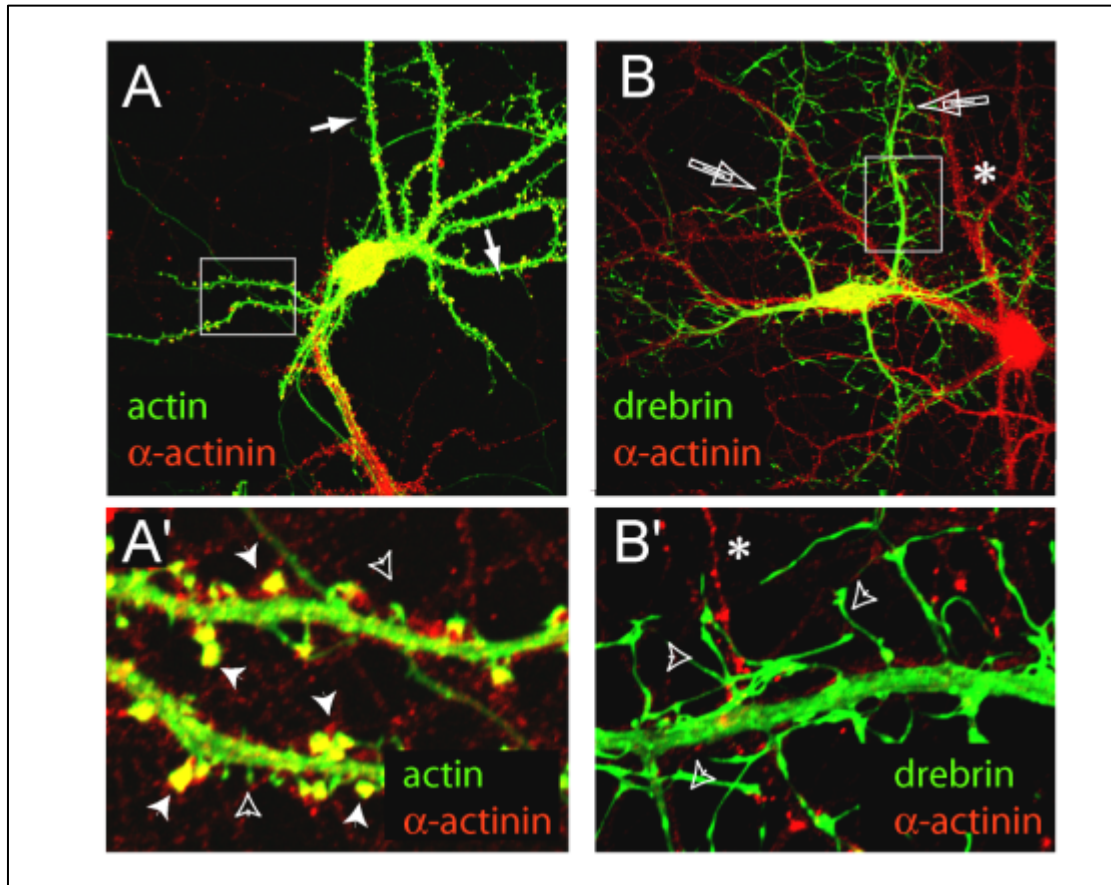


Figure 12: Drebrin displaces a-actinin from mature dendrite:

Single plane from confocal image from (A-A') GFP-actin and (B-B') drebrin AIRE-GFP-actin transfected neurons, fixed after 21DIV and stained with anti-a-actinin antibody. (A-B') a-actinin was associated to dendritic protrusions of control neurons (A arrows; A' arrowheads) but was absent from such structures in drebrin A over expressing cells (B open arrows, B' open arrowheads; asterisks point towards the dendrite of an untransfected neighboring neuron expressing a-actinin.)

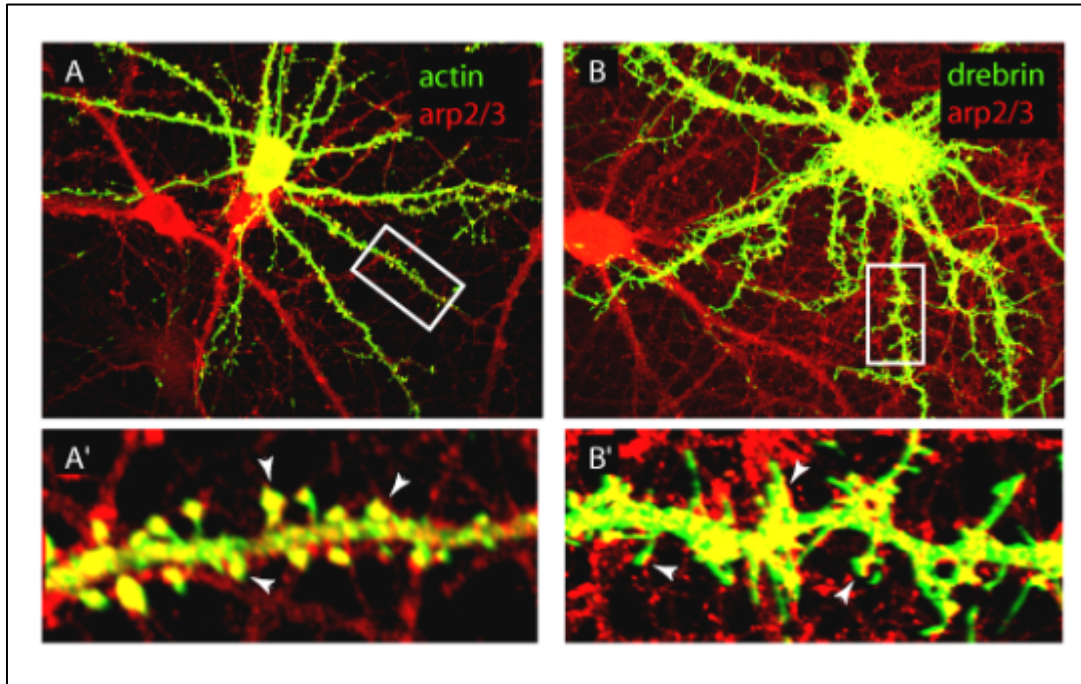


Figure 13: The Arp2/3 complex colocalizes with dendritic protrusions in GFP-actin and drebrin A-GFP transfected:

Single plane from confocal image from (A-A') GFP-actin and (B-B') drebrin A-IRES-GFP-actin transfected neurons, fixed after 21DIV and stained with p-34-arc antibody. (A-B'). The Arp2/3 complex is associated with dendritic protrusions on control GFP-actin transfected neurons (A; A' closed arrows) and drebrin A-IRES-GFP-actin transfected neurons (B, B' closed arrows).

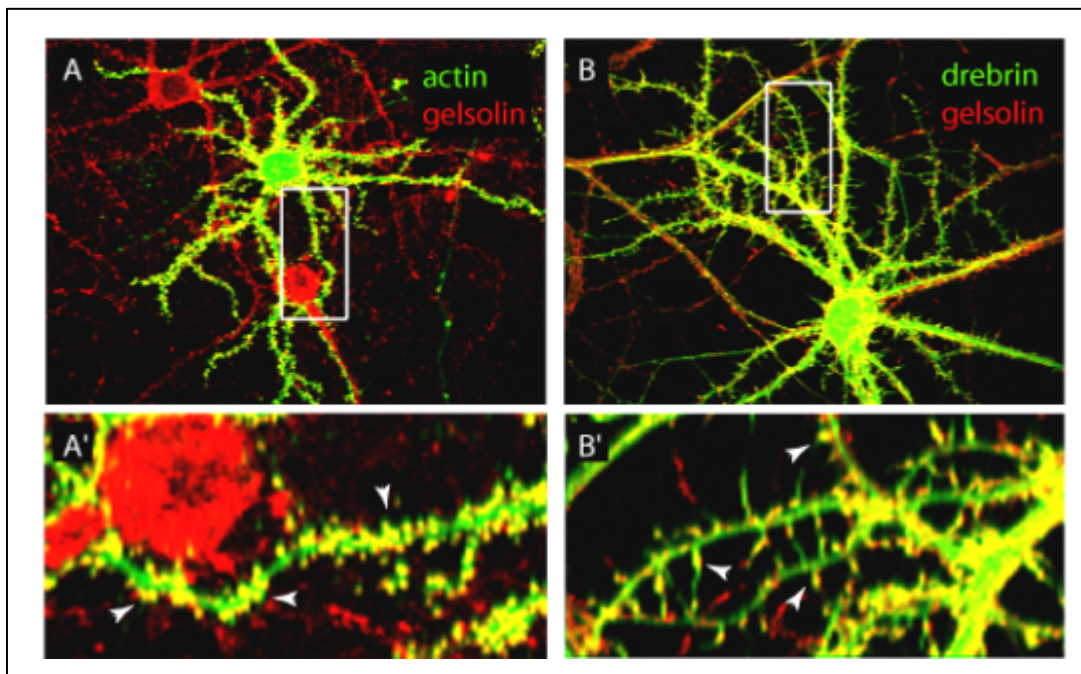


Figure 14: Gelsolin localization is unchanged in drebrin A overexpressing neurons:

Single plane from confocal image from (A-A') GFP-actin and (B-B') drebrin A-IRES-GFP-actin transfected neurons, fixed after 21DIV and stained with gelsolin antibody. (A-B'). Gelsolin is strongly associated with dendritic protrusions on control GFP-actin transfected neurons (A; A' closed arrows) and drebrin A-IRES-GFP-actin transfected neurons (B, B' closed arrows).

DISCUSSION:

To date, there is little known about the molecular mechanisms that maintain and regulate the structure of mature dendritic spines. It is nevertheless clear that the characteristic narrow neck, expanded head shape of mature spines depends on the arrangement of actin, which is the predominant component of the spine cytoskeleton (Kaeck et al., 1997; Matus et al., 1982). Electron microscopy studies have indicated a partitioning of actin filaments within the spine cytoskeleton with long filament bundles extending from the spine neck into the core of the head region whereas the head periphery contains irregularly oriented small bundles and single filaments (Cohen et al., 1985; Landis and Reese, 1983). This arrangement is consistent with live imaging experiments using GFP-tagged actin which show dynamic actin in the head periphery producing rapid surface motility at the tips of spines while over periods of up to 2 hours the basic structures of both the neck and the head do not vary (Fischer et al., 1998; Star et al., 2002).

The three-dimensional structure of such actin filament networks as well as their relative stability or motility is determined by a set of actin binding proteins controlling the length, the stability and the strength of an individual actin filament as well as the number and pattern of inter-filament attachments (reviewed in (Ayscough, 1998).

This study shows that overexpression of drebrin is sufficient to lead to the re-emergence of filopodia and protospines-like structures and to trigger enhanced morphological plasticity of dendritic protrusions. Overexpressing drebrin even after synapses had attained their mature morphology resulted in the same effect, implying a role of drebrin in regulating the stability of mature adult spines.

However, whereas drebrin is important for controlling the fundamental structural stability of dendritic spines, it is not involved in mediating the rapid surface motility of the spine head which can be blocked by activating glutamate receptors (Fischer et al., 2000). Our results thus demonstrate that the structure of mature dendritic spines is a complex of at least two independent molecular mechanisms, one that regulates surface motility and one that involves drebrin and actin-bundling proteins to maintain the fundamental narrow neck, expanded head configuration necessary for spines to

fulfill their role as biochemical compartments. This situation is reminiscent of the partitioning of the actin cytoskeleton in crawling fibroblasts where dynamic actin filaments drive surface ruffling at the leading edge while more stable actin filament bundles participate in maintaining the shape of the cell body.

Even though protrusions from dendrites overexpressing drebrin displayed high morphological plasticity, polymorphic spines stay attached to presynaptic terminals. Glutamate receptors, as well as scaffolding proteins, also remained clustered in destabilized polymorphic spine heads, thus drebrin overexpression did not induce dissolution of synaptic structures in polymorphic spines further indicating the functional differentiation of multiple molecular mechanisms determining dendritic spine structure.

It has been previously shown that molecular constituents at mature synapses are largely independent of F-actin. In contrast to N-cadherin clusters, bassoon, NMDA-receptors and PSD-95 clusters could not be dissipated by prolonged latrunculin A treatment (Allison et al., 2000; Zhang and Benson, 2002). This particular stability is believed to be established through the strong protein-protein interactions between scaffolding proteins and to binding to transmembrane molecules and receptors creating a robust matrix. AMPA-receptor cluster formation, in contrary, had previously been linked to an intact actin cytoskeleton and is prevented by latrunculinA treatment (Allison et al., 1998). This dependence is plausibly linked to the fast AMPA-receptor turnover occurring at excitatory sites and does not appear to be influenced by drebrin-induced destabilization of postsynaptic actin arrays.

However, drebrin-induced filopodia were never innervated nor did they accumulate postsynaptic molecule clusters, thus there seems to be a critical point where, despite their large independence to F-actin, postsynaptic clusters can no longer be maintained.

How does drebrin induce destabilization of spine structures? Drebrin has been shown to have no severing or crosslinking activity, instead it is involved in regulating the binding of the actin stabilizing proteins fascin (Sasaki et al., 1996), α -actinin and tropomyosin (Ishikawa et al., 1994) to F-actin. These proteins are generally involved in organizing F-actin into supramolecular structures associated with stable actin

networks. We have demonstrated that the unique actin binding domain of drebrin is both necessary and sufficient to induce structural disruption of dendritic spines, suggesting that the competition between drebrin and actin cross-linking proteins is responsible for the observed remobilization of dendritic protrusions. In agreement with that idea, drebrin overexpression might be accompanied by a delocalization of stabilizing proteins and indeed we find that high levels of drebrin corresponds to a general down-regulation of the actin crosslinking protein α -actinin in neurons. Furthermore, the localization of two other molecules involved in fast actin polymerization, the arp2/3 complex and gelsolin, remains unchanged. Therefore our results imply a fundamental importance for supramolecular actin networks in determining integral spine stability and provide evidence for a major role of drebrin in adjusting the level of stable actin within a spine head. We propose the following model for destabilization of spine structure. Both drebrin and actin cross-linking proteins exist as components of the actin cytoskeleton at the postsynapse. Upon signals which remain to be determined, either the fraction of cross-linking proteins bound F-actin increases leading to an overall consolidation of dendritic spine structure, or the portion of drebrin bound F-actin rises with the consequence of a collapse of the actin networks and focal adhesions corresponding to a destabilization of dendritic spine structures (Figure 15).

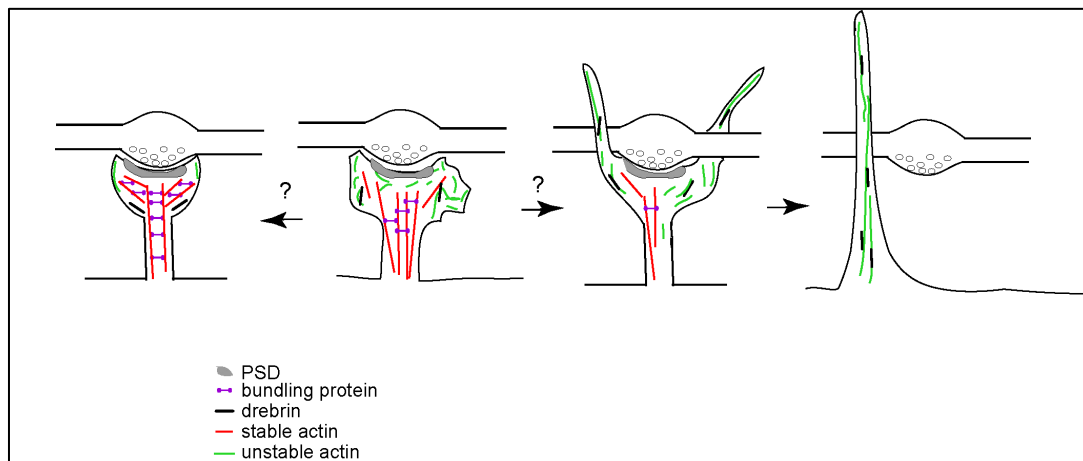


Figure 15: Model for drebrin regulated dendritic spine stability:

Stable, cross-linked actin filaments and dynamic, drebrin-bound, actin filaments coexist within dendritic spines. Upon Ras activation, the ratio of drebrin-bound actin increases which concomitantly elicits a destabilization of spine structure leading to the formation of polymorphic spines and filopodia. Conversely when Ras is downregulated, bundled actin predominate, thus stabilizing dendritic spine structure.

Part III**REMODELING OF THE ACTIN CYTOSKELETON BY DREBRIN IS
REGULATED BY THE MAP-KINASE PATHWAY**

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SUMMARY

Ras proteins are small GTP-ases with well known functions in cell proliferation and differentiation. They play a key role as molecular switches that can trigger distinct signal transduction pathways, such as the mitogen-activated protein (MAPK) pathway or the phosphoinositide-3 kinase pathway. Besides controlling gene transcription, Ras has been implicated in the regulation of the release of adhesion.

Furthermore, activation of the Ras-MAPK pathway has been shown to destabilize dendritic spine structure in mature neurons. Here we demonstrate that the Ras-MAPK pathway is involved in regulating the association of drebrin to actin. Thus we propose, that the structural stability of dendritic spines is governed by the ratio of drebrin bound to actin, a ratio that is controlled by the MAPK pathway.

INTRODUCTION

Small specialized dendritic protrusions, termed dendritic spines, harbor the majority of excitatory synapses in the adult brain. Dendritic spines are believed to influence synaptic transmission in various ways (Hausser and Roth, 1997; Svoboda et al., 1996), and have been furthermore shown to be highly plastic with both rapid inherent motility (Dunaevsky et al., 2001; Fischer et al., 1998; Star et al., 2002) and slower changes consisting of substantial structural changes such as shrinkage or outgrowth (Engert and Bonhoeffer, 1999; Trachtenberg et al., 2002) having been reported.

The underlying cytoskeletal component of dendritic spines is composed of actin networks which are conceivably responsible for this morphological plasticity (Kaech et al., 2001; Matus et al., 1982). Indirect evidence points towards two actin pools within dendritic spines; a highly dynamic one close to the surface membrane, driven by fast actin turnover and a stable one characterized by slow actin turnover (Allison et al., 1998; Fischer et al., 1998).

We have shown previously that overexpression in dissociated hippocampal neurons of an actin binding protein, drebrin, which interferes with F-actin bundling, destabilizes dendritic spines and elicits the outgrowth of filopodia and highly plastic polymorphic spines from mature dendrites. The morphological alterations induced by drebrin could also be generated by overexpressing a construct containing only the unique actin binding domain of drebrin indicating that drebrin's effect depend on its ability to compete with other actin binding proteins which bundle actin. Thus it appears that the structural stability of dendritic spines depends in part on maintaining a balance between the binding of drebrin and of other actin stabilizing proteins to actin filaments in the spine cytoplasm.

How might this differential binding be regulated? It had been previously shown that activation of Ras by spaced stimuli or BDNF treatment results in filopodial outgrowth from mature dendrites reminiscent of the phenotype induced by drebrin overexpression (Wu et al., 2001). Importantly, these effects are MEK1 dependent. Furthermore, activation of the EphB2 receptor, which had been shown to cause the downregulation of the Ras-mitogen activated protein kinase (MAPK) pathway (Elowe

et al., 2001) stabilizes spine structure and even triggers premature spine formation in hippocampal neurons (Ethell et al., 2001). We therefore considered the Ras-MAPK pathway to be a candidate for controlling drebrin binding to F-actin.

To address this question, we first studied Ras dependent regulation of drebrin in non-neuronal cells, rat embryonic fibroblasts (REF-52), which have a well defined actin cytoskeleton and therefore offer themselves as ‘test-tube’ to study the regulation of actin filaments assembly.

The experiments presented here show that drebrin overexpression triggers retraction of the cytoplasm, a phenomenon accompanied by stress-fibers collapse and dissipation of focal adhesion, reminiscent of Ras transformation (Matsumoto et al., 1997). Drebrin induced cell retraction was partially rescued by dominant negative Ras mutants, an effect dependent on the C-terminal part of drebrin, containing a polyproline-rich sequence and an SH3 domain. Furthermore we show that the subcellular localization of endogenous drebrin is regulated by Ras activation and that drebrin binds to actin in a MEK dependent fashion in REF-52 cells.

Finally, constitutively active Ras triggered outgrowth of filopodia and polymorphic spine on dendrites of mature neurons and dominant negative Ras was capable of blocking drebrin-induced dendrite phenotype. Taken together these results reveal a Ras-dependent mechanism for the stabilization of dendritic spines.

RESULTS

Drebrin overexpression induces cell retraction in REF-52 fibroblasts

Drebrin overexpressing fibroblasts reportedly exhibit gross morphological distortion resulting in cell morphologies formerly said to be neuron-like. This phenotype was proposed to be the consequence of excessive growth of cell processes (Shirao et al., 1992) but in fact would be more consistent with retraction of the cytoplasm since overall cell diameter does not appear to change following drebrin overexpression. To choose between these interpretations, we transfected rat embryonic fibroblast with a construct encoding GFP-tagged drebrin (drebrin-GFP) and measured the surface area occupied by individual drebrin overexpressing cells compared to GFP-actin control cells. As seen in Figure 1 A, drebrin overexpression resulted in a variety of phenotypes ranging from cells displaying normal spread shapes, intermediate, that is partial cellular retraction, or completely retracted profile. When the total surface area of these cells was calculated we found that drebrin-modified fibroblasts occupy the same or less surface area as do GFP-actin transfected control cells (Figure 1B). It is thus clear that the observed drebrin-induced phenotypes in fibroblasts are the result of cell retraction and not of disproportionate process outgrowth.

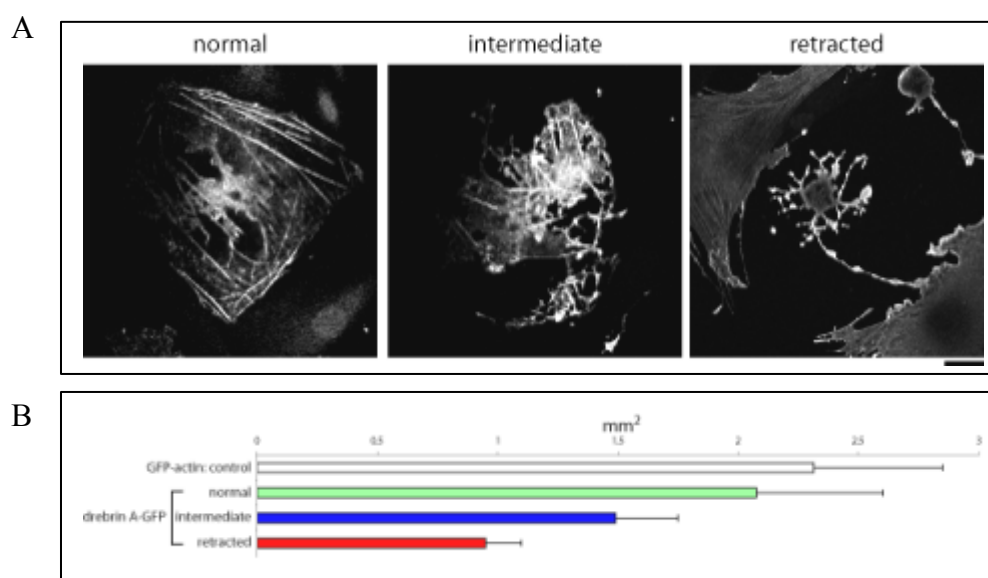


Figure1: Drebrin overexpression leads to cell retraction in REF-52 cells:

(A) Confocal images of REF-52 cells transfected with drebrin-GFP showing examples of cells displaying normal spread, intermediate or retracted phenotype.

(B) The graph shows the mean surface area occupied by drebrin-GFP overexpressing fibroblasts displaying normal spread (green), intermediate (blue) or retracted (red) phenotype as compared to GFP-actin transfected control fibroblasts (white). Means were calculated from 10 individual cells displaying the described phenotypes and from ten control GFP-actin transfected cells.

Drebrin triggers stress fibers breakdown and dissipation of vinculin clusters in REF-52 fibroblasts

How does drebrin induce the observed retraction phenotypes? Cell retraction is typically associated with the collapse of its underlying cytoskeleton. As drebrin is known to interfere with α -actinin and tropomyosin binding to F-actin (Ishikawa et al., 1994) and since both proteins are involved in the formation and maintenance of stable actin filament networks in cells, such as stress fibers in fibroblasts (Morgan and Gangopadhyay, 2001), we addressed the effect of drebrin on actin filament networks in rat embryonic fibroblasts (REF-52) cells by transfecting them with a construct encoding HA-drebrin and staining them after 48 hours with an HA-specific antibody to visualize overexpressed drebrin and Rhodamine-phalloidin to selectively visualize F-actin.

Figure 2 shows three representative REF-52 cells expressing HA-drebrin displaying normal spread, intermediate or retracted shape. As illustrated in the upper row, cells displaying a normal phenotype have well defined stress fibers spanning the entire cytoplasm. Overexpressed drebrin partially colocalizes with stress fiber and especially with the actin based cell cortex, however, a substantial amount is diffusely distributed throughout the cytoplasm.

As seen in the middle and lower panel, increasing degrees of retraction are associated with a concomitant collapse of stress fibers gradually collapse concomitantly with cell retraction which are virtually absent from fibroblasts displaying complete cell retraction. In these retracted cells, drebrin is increasingly localized to the membrane but, despite the obvious collapse of actin-based networks, strong phalloidin staining is still detectable in retracted cells, indicating that drebrin does not cause actin depolymerization, but rather triggers the reorganization of actin networks.

Drebrin competes with α -actinin, which besides configuring stress-fibers, participates in anchoring actin filaments to the extracellular matrix by binding to vinculin, a component of cell adhesion complexes (Miyamoto et al., 1995; Shakibaei et al., 1999). The submembranous localization of drebrin together with its effects on cell morphology, suggests that drebrin might affect cell adhesion. Consequently, we

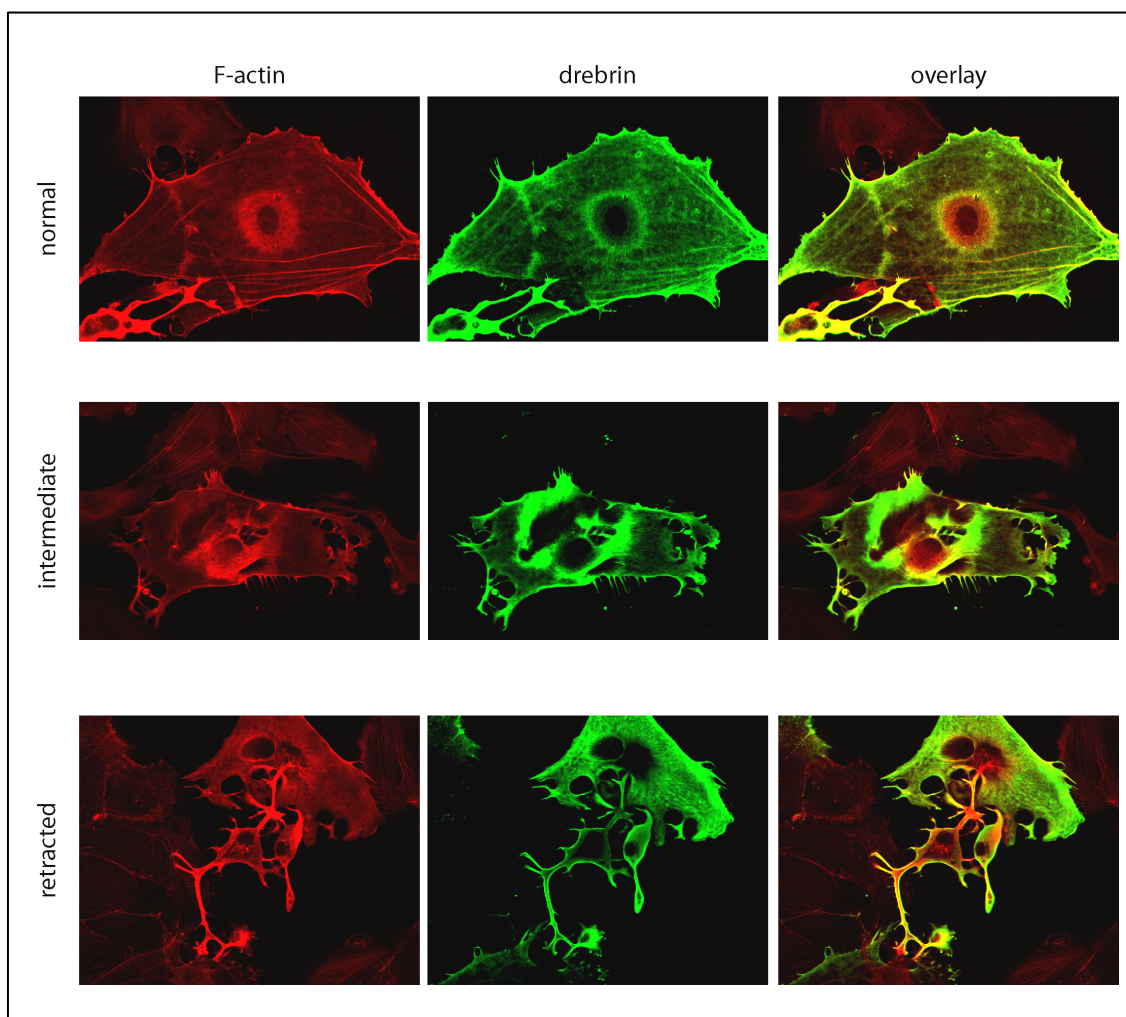


Figure2: Drebrin overexpression induces collapse of stress fibers in REF-52 fibroblasts:

Confocal images showing F-actin (red) in HA-drebrin (green) transfected REF-52 cells displaying normal, intermediate or retracted phenotype. Drebrin partially colocalizes with stress fibers and is especially associated with cortical actin. Stress fibers collapse concomitantly with cell retraction and are absent in cells displaying a retracted phenotype. However, F-actin is still present in retracted fibroblasts, as seen by phalloidine staining. Left column: F-actin. Middle column: HA-drebrin. Right column: Overlay; drebrin=green, F-actin=red.

examined the state of adhesion plaques in drebrin overexpressing fibroblasts. Adhesion plaques were visualized by staining drebrin-GFP transfected fibroblasts with an antibody directed against vinculin 48 hours after transfection.

Figure 3 illustrates the dissipation of vinculin clusters, which parallels drebrin-induced cell retraction. In normal spread fibroblasts, vinculin is organized in distinct clusters at the end of stress fibers. However, fibroblasts showing different degrees of cytoplasmic retraction also show a corresponding loss of the well-defined assembly of

vinculin, characteristic of control cells. In retracted cells, vinculin is diffusely distributed in the cytoplasm, indicating that drebrin-induced cell retraction is accompanied by the loss of focal adhesionsites.

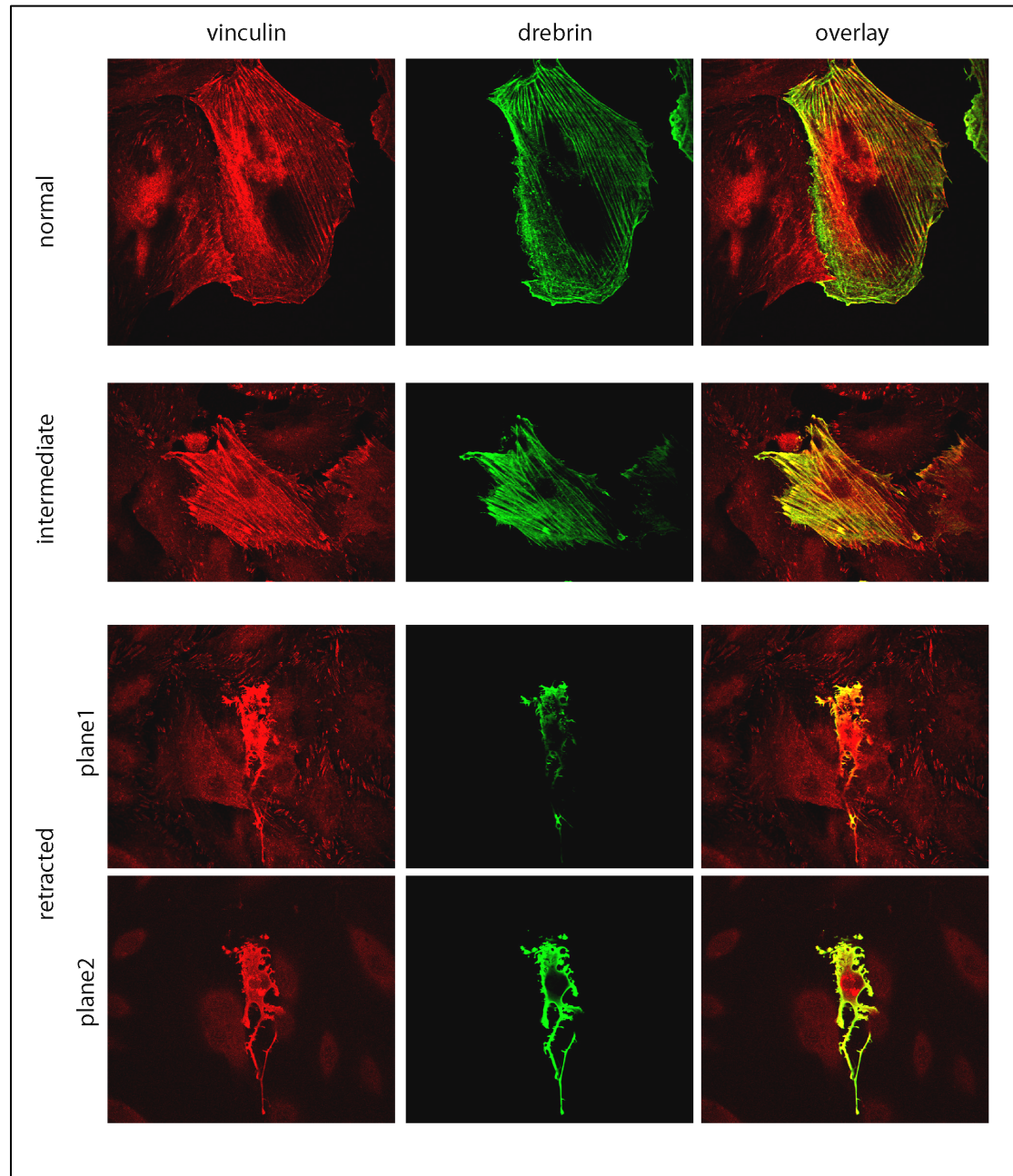


Figure 3: Drebrin overexpression in REF-52 cells leads to dissipation of vinculin clusters:

Confocal planes showing vinculin (red) in drebrin-GFP (green) transfected REF-52 cells displaying normal, intermediate or retracted phenotypes. In normally spread cells, vinculin is assembled in distinct clusters similar to neighboring untransfected cells. As cell retraction increases, vinculin clusters become progressively dissipated. Vinculin clusters are absent in cells displaying the fully retracted phenotype. However, vinculin signal is present in retracted fibroblasts, where it is distributed diffusely throughout the cytoplasm as seen in retracted plane1 and plane 2.

Interactive effects of Ras and drebrin on fibroblast morphology

High levels of drebrin induce stress fiber collapse and dissipation of vinculin clusters in REF-52 cells, strongly resembling the phenotype observed in Ras transformed cells (Shah et al., 2001; Tikoo et al., 1994). Moreover, the Ras-MAPK pathway has been implicated in release of adhesion and disassembly of stress fibers at the trailing edge of migrating fibroblasts (Fernandez-Patron et al., 2002; Lu et al., 1998) pointing towards a possible relationship between Ras and drebrin.

Drebrin and constitutively active Ras have synergic effects on fibroblasts morphology:

We tested the possible relationship of drebrin by cotransfecting REF-52 cells with drebrin and constitutively active or dominant negative mutants of Ras. We used mutants of two Ras isoforms for these experiments; constitutively active (K-Ras V12) and dominant negative (K-Ras N17) form of the ubiquitously expressed K-Ras (Zhang and Woloschak, 1998) or the constitutively active (M-Ras V22) and dominant negative (M-Ras N27) M-Ras mutants of the brain specific Ras isoform M-Ras (Matsumoto et al., 1997), together with GFP-actin. Control cells were transfected with GFP-actin alone. Cells were fixed 48 hours after transfection and their morphology assessed. A total of 1500 cells from 4 transfection experiments were quantified in this experiment.

As illustrated in Figure 4, drebrin and constitutively active forms of Ras were equally capable in inducing cell retraction in REF-52 fibroblasts. When drebrin was expressed together with constitutively active Ras, both proteins had synergic effects on cell morphology, elevating the ratios of retracted cells to 60-80%.

Dominant negative Ras blocks drebrin-induced cell retraction:

Dominant negative Ras, on the contrary, had no effect on cell morphology. Coexpression with dominant negative isoforms of Ras in contrast, blocked the drebrin-induced retraction phenotype elevating the ratios of normal cells to 50%, pointing toward a possible relationship between Ras induction and drebrin function.

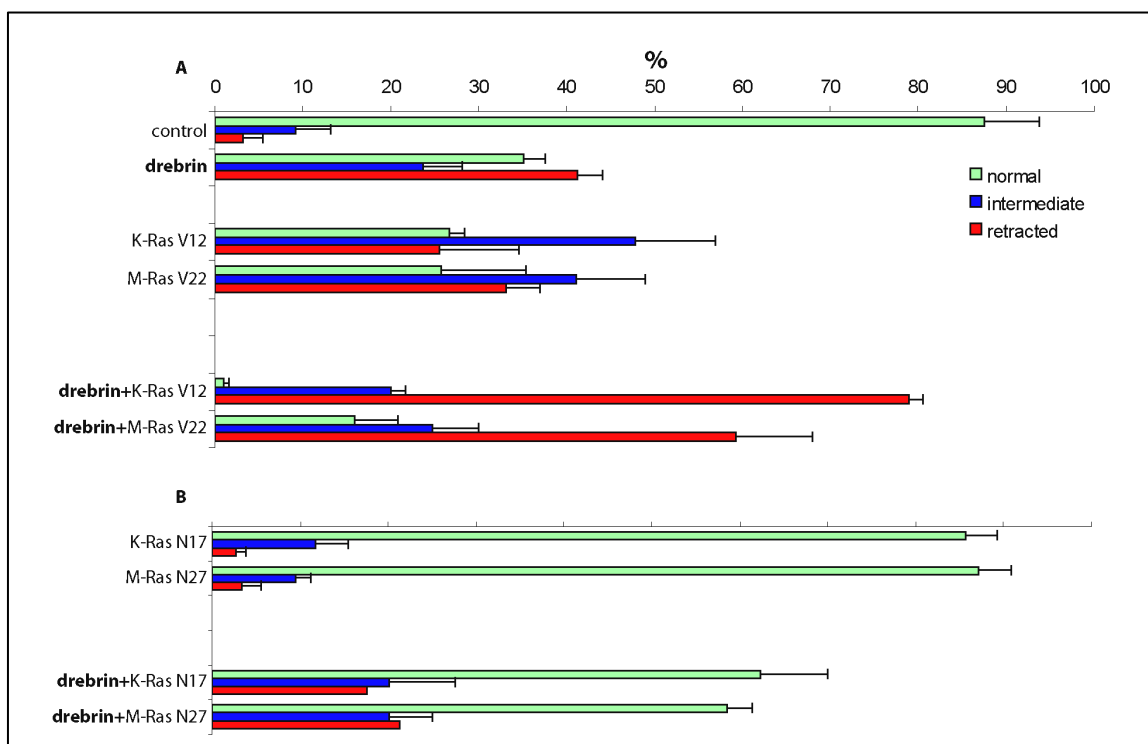


Figure 4: Drebrin and constitutively active Ras have synergic effect on cell morphology and dominant negative Ras blocks drebrin-induced cell retraction:

The graph shows the mean percentage of the three different cell morphologies displayed by fibroblasts transfected with Ras mutants, drebrin or combinations of the two proteins compared cells transfected with GFP-actin as a control. Both constitutively active Ras mutants and drebrin were equally effective in promoting cell retraction. When cotransfected, constitutively active Ras and drebrin showed a synergic effects pushing the percentage of retracted cells above 55%. Overexpression of dominant negative Ras did not trigger morphological changes in fibroblasts. Cotransfection of drebrin and dominant negative Ras of either isoforms induced a significant increase percentage of normal spread cells and significantly reduced the proportions of retracted cells.

However, the synergistic effects of constitutively active Ras and the blockade of dominant negative Ras on drebrin-induced cell retraction might involve two distinct pathways. To determine whether drebrin lies downstream of Ras, we cotransfected REF-52 fibroblasts with drebrin or drebrin mutants together with dominant negative Ras isoforms along with GFP-actin and assessed cell morphology as compared to GFP-actin transfected control cells.

Figure 4A illustrates the different drebrin mutants used in this experiment. All deletion mutants were capable of inducing cell retraction, however the C-terminal mutant containing a polyproline rich sequence though to be responsible for drebrin's association to profilin, a SH3 domain and an actin binding site unique to drebrin as well as a deletion mutant containing this unique actin binding site alone were less effective than full length drebrin. The N-terminal mutant however containing both actin binding sites of drebrin was as efficient as full length drebrin. Yet, only cell retraction induced by the C-terminal mutant of drebrin could be blocked when

coexpressed with dominant negative Ras mutants indicating that drebrin lays indeed downstream of Ras and that the C-terminal is responsible for the regulation of drebrin function.

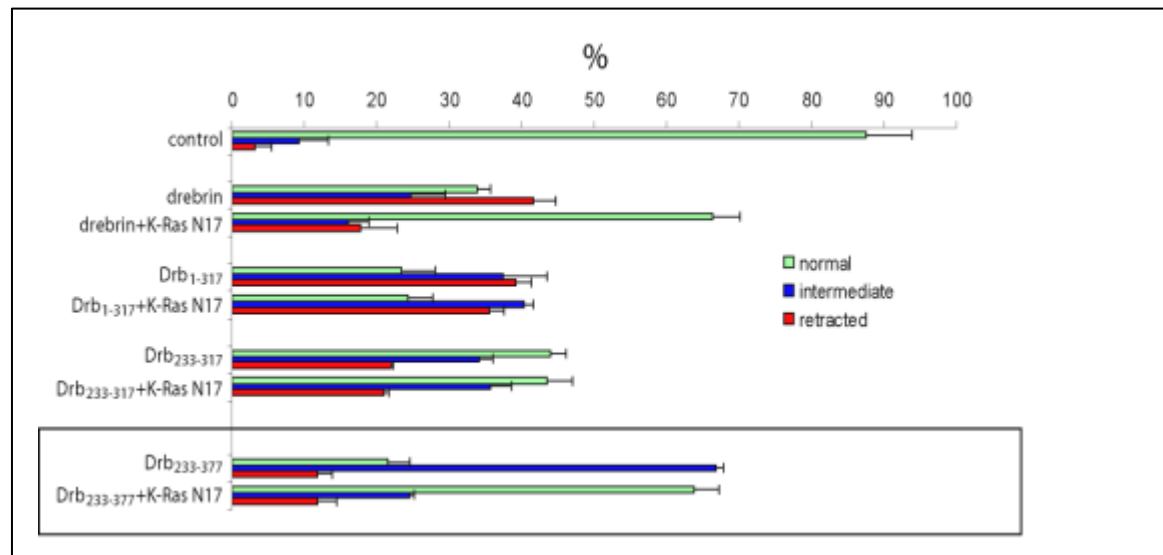


Figure 5: The C-terminus of drebrin is required for blockade of cell retraction by dominant-negative Ras:

A) Schematic overview of drebrin mutants used in this experiment.

B) The graph shows the mean percentage of the three different cell morphologies displayed by fibroblasts transfected with either drebrin or drebrin mutants alone, or in combination with dominant negative Ras compared to REF-52 cells transfected with GFP-actin as a control. Only drebrin and Drb 233-707 induced cell retraction could also be blocked by dominant negative Ras.

Drebrin binding to actin is regulated by the Ras-MAP-kinase pathway in REF-52 cells

How is drebrin function affected by Ras? It appears that drebrin association to F-actin and the subsequent displacement of cross-linking proteins and collapse of supramolecular F-actin networks (Hayashi et al., 1999; Ishikawa et al., 1994) appears to be a key step for drebrin-induced cell retraction of fibroblast and formation of polymorphic, motile structures on neuronal dendrites (see part II). Therefore, a possible mechanism by which Ras may control drebrin function could comprise the regulation of actin-drebrin association. To tackle this question we stimulated quiescent REF-52 cells with EGF to activate Ras for 30 minutes before fixation and stained them subsequently with a drebrin antibody to visualize endogenous drebrin. In parallel experiments, we averted EGF induced Ras stimulation with the Ras inhibitor Farnesylthiosalicylate (FTSCA), which specifically blocks Ras farnesylation and therefore activation (Levitzki, 1996).

As demonstrated in Figure 6, endogenous drebrin is diffusely distributed around the nucleus in quiescent fibroblasts (Figure 6 left panel). This is in agreement with previous reports, showing that drebrin does not often colocalize with F-actin but is instead diffusely distributed within the cytoplasm or organized to unidentified intracellular dots in resting non neuronal cells (Peitsch et al., 2001). Upon EGF stimulation, endogenous drebrin was strongly colocalized with stress fibers, arguing in favor of a Ras mediated actin-drebrin interaction (Figure 6 middle panel). When Ras activation by EGF was blocked by FTSCA, however, this did not only prevent drebrin targeting to stress fibers, but in addition, triggered redistribution of drebrin into small intracellular spots (Figure 6, right panel). However, thus far we could not identify the nature of these small dots.

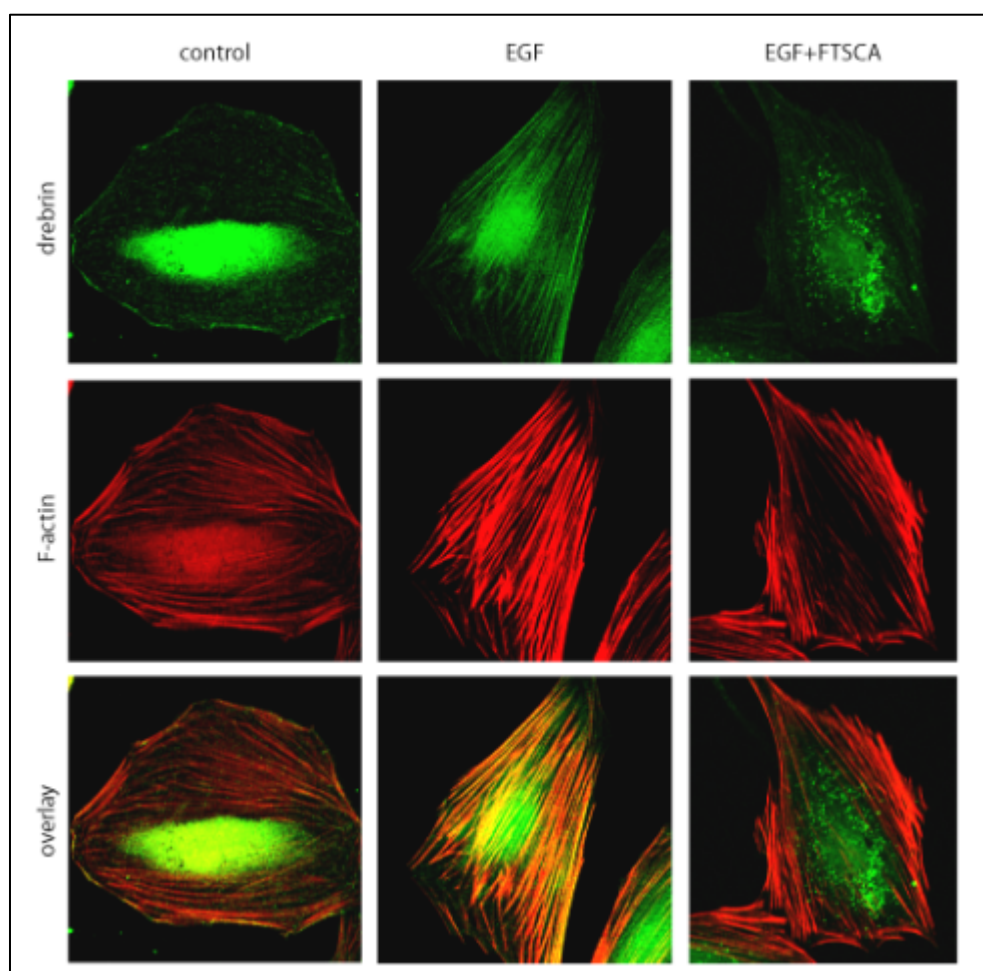


Figure 6: Drebrin is localized to stress fibers upon EGF stimulation

Upper panel: Distribution of endogenous drebrin in quiescent cells or upon treatment with EGF or EGF+FTSCA. Middle panel: F-actin in the same cells, visualized by Rhodamine-phalloidin. Lower panel: merge; drebrin=green, F-actin=red. In quiescent fibroblast, drebrin is diffusely distributed in the cytoplasm and concentrated around the cell nucleus or at cell borders. EGF treatment redistributes drebrin to stress fibers as illustrated in the middle column. Blockage of Ras by FTSCA triggers the redistribution of drebrin into small intracellular dots (left column)

We next asked whether drebrin binding to F-actin is itself regulated by Ras. To examine this possibility we immunoprecipitated drebrin from quiescent and EGF-stimulated fibroblasts. In parallel we treated REF-52 cells with EGF plus FTSCA, the IP3-kinase inhibitor LY-294002 or the MAP-kinase inhibitor U0296. Samples were analyzed by Western blotting and stained with drebrin and actin antibodies. As seen in Figure 7, EGF stimulation augmented the amount of actin that could be pulled down along with drebrin. Blocking Ras farnesylation with FTSCA or blocking the MAP-kinase pathway with of the specific MEK inhibitor U0296 completely abolishes drebrin-actin association. In contrast, blocking IP3-kinase with of the specific blocker LY294002, does not influence the binding of drebrin to actin.

We conclude that drebrin binding to actin is regulated by Ras and depends on activation of MEK-1.

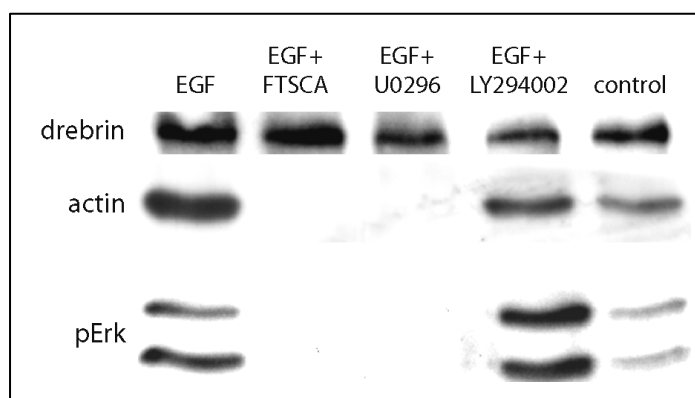


Figure 7: Drebrin-actin association is governed by the MAP-kinase pathway in REF-52 cells.

REF-52 cells were seeded onto 6mm dishes and serum starved for 24 hours prior to stimulation with 10ng/ml EGF with or without the blockers FTSCA, U0296 or LY294002 (all 10 μ M) for 30 min. Cells were lysed and drebrin was immunoprecipitated using a specific drebrin antibody. Drebrin-actin association is enhanced by Ras activation. Drebrin does not bind to actin after selective blockage of MEK1.

Constitutively active Ras induces morphological changes in dendritic protrusions comparable to those induced by drebrin.

The brain is the most important source of drebrin in an adult mammal. As described in part II, drebrin overexpression in adult neurons leads to the outgrowth of filopodia and polymorphic spines from mature dendrites. Since drebrin lies downstream of Ras

and the drebrin-actin association is regulated by the MAPK pathway in fibroblasts, we next examined whether this could also be the case in hippocampal neurons.

To do this we transfected dissociated hippocampal neurons with a bicistronic vector encoding GFP-actin and constitutively active or dominant negative forms of Ras either alone or in combination with drebrin. The cultures were fixed after three weeks, when neurons have fully matured in vitro and cell morphology analyzed.

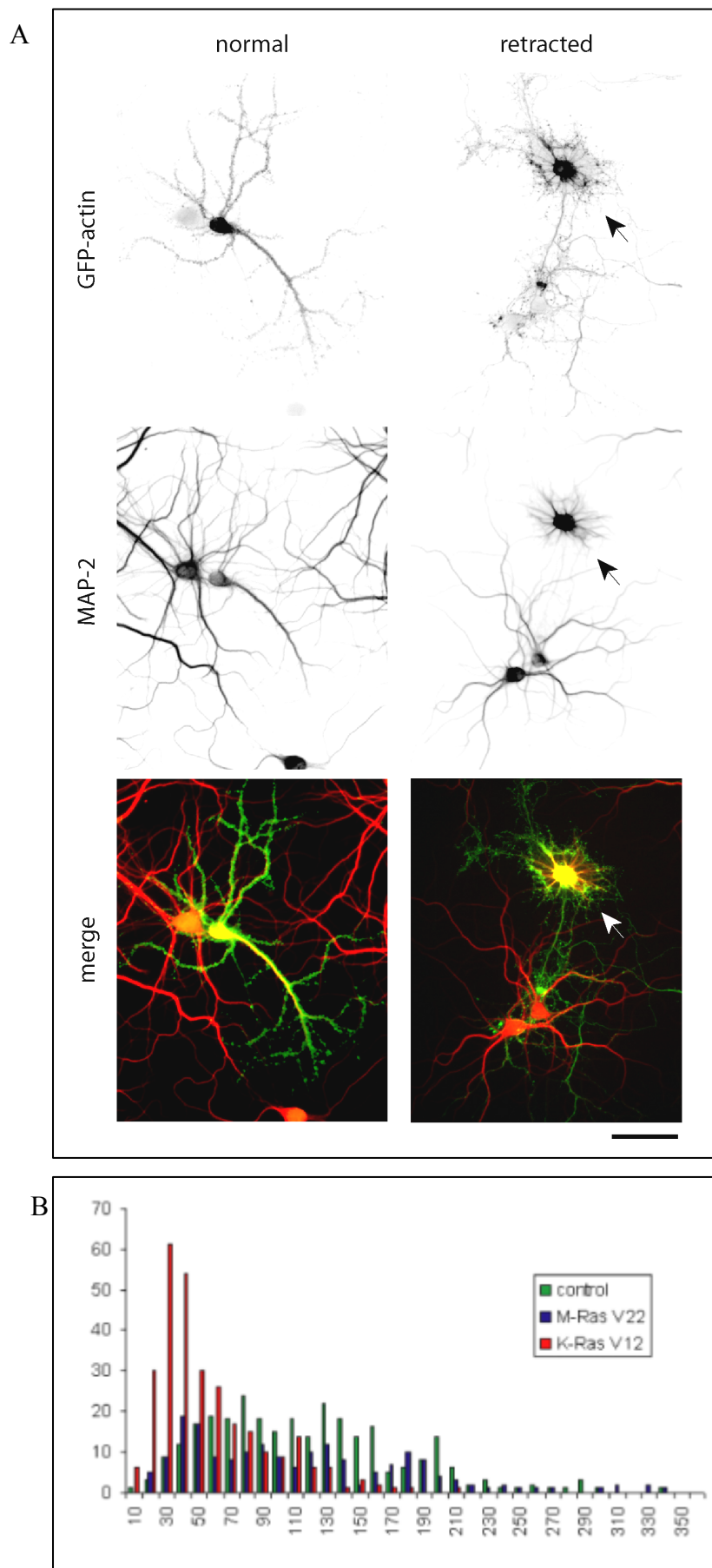
Dominant negative Ras had no detectable effect on neuron morphology. Neurons transfected with either construct encoding dominant negative Ras, K-Ras N17 or M-Ras N27, build up dendritic trees of normal appearance with dendrites extending and arborizing in a fashion comparable to GFP-actin transfected control neurons, demonstrating that blocking Ras function during development has no effect on dendrite development.

Constitutively active forms of Ras, in contrast, elicited dendrite retraction in the majority of transfected hippocampal neurons, an effect strongest observed in K-Ras V12 overexpressing neurons (Figure 8 A and B). In addition, dendritic trees of those cells were also highly complex, dendrites branching extensively, as assessed by counting total numbers of dendrite ends per neuron (Figure 8). It appears therefore that expression of activated Ras throughout development has a profound effect on dendrite extension.

Furthermore, when constitutively active Ras mutants were transfected together with drebrin very few neurons survived past three weeks. From two different experiments, only 16 neurons double transfected with constitutively active Ras and drebrin survived three weeks in vitro versus ca. 280 GFP-actin transfected control neurons, suggesting that the total breakdown of the cytoskeleton is lethal for dissociated hippocampal neurons. The few remaining neurons displayed gross morphological changes including dendrite retraction and enlargement of cell bodies.

Figure 8: Constitutively active Ras triggers dendrite retraction in dissociated hippocampal neurons:

(A) Upper row: Dissociated hippocampal neurons transfected with K-Ras V12 /IRES/ GFP-actin showing normal or retracted morphology. Middle row: MAP2 counterstain identifying both cells as neurons. Lower row: merge; GFP-actin=green, MAP2=red. Scale bar= (B) The graph shows the distribution of dendrite length in control GFP-actin versus M-Ras V22/IRES/ GFP-actin and K-Ras V12/IRES/ GFP-actin transfected neurons.



Besides its profound effect on dendrite elongation, expression of constitutively active Ras also influenced the morphology of dendritic protrusions.

While dendritic protrusions on control GFP-actin transfected neurons grew spines of mature appearance, characterized by a bulbous head connected to the dendritic shaft by a narrow neck (Figure 9A, top row and 9B), protrusions on dendrites of K-Ras V12 or M-Ras V22 displayed highly destabilized morphologies comparable with dendritic protrusions of drebrin overexpressing cells. First, the amount of filopodia was clearly increased on these cells as compared to control neurons and second spiny protrusions often displayed irregular shapes reminiscent of those elicited by drebrin overexpression (Figure 9A, middle row and 9B).

Dominant negative Ras, in contrary, had no noticeable effect on the morphology of dendritic protrusions, which could not be discriminate from those on control GFP-actin transfected neurons (Figure 9A, top row and 9B).

Finally, when drebrin and dominant negative Ras isoforms were transfected together, drebrin-induced formation of filopodia and outgrowth of polymorphic spines was partially blocked. On these neurons outgrowth of filopodial protrusions was markedly reduced (Figure 9A, bottom row and 9B). However, a high proportion of dendritic protrusion in those cells still displayed irregular shapes (Figure 9B) suggesting that a shift from filopodia to polymorphic spines had occurred.

Thus it appears that, like in fibroblasts, drebrin-induced destabilization of stable actin arrays is controlled by Ras in dissociated hippocampal neurons. However further experiments will be required to confirm that drebrin-actin association is regulated by Ras in mature neurons.

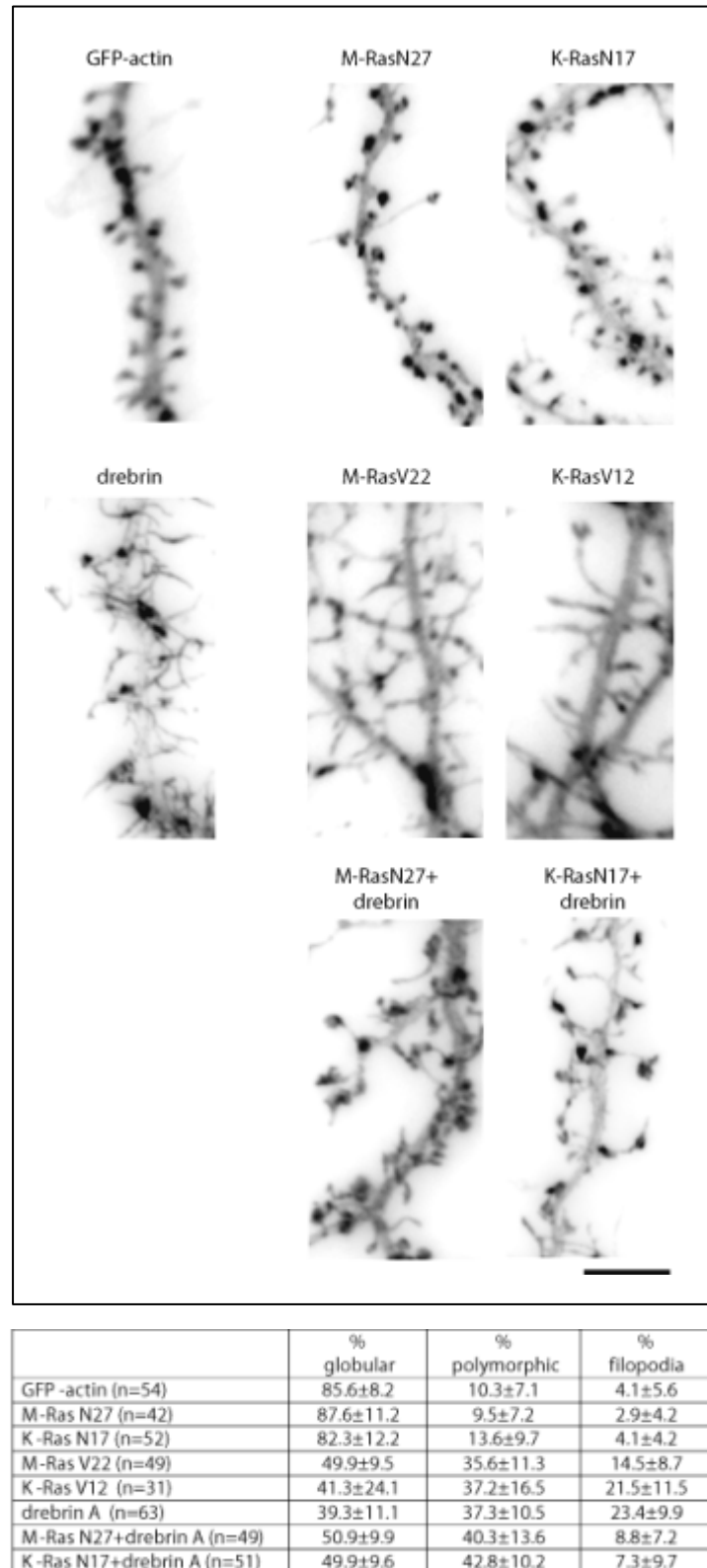


Figure9: Constitutively active Ras and drebrin have similar effects on dendritic protrusions and dominant negative Ras partially blocks drebrin-induced phenotype

(A) Dendrites of neurons transfected GFP-actin, drebrin A, constitutively active and dominant negative forms of Ras alone and dominant negative isoforms of Ras together with drebrin A. (B) The table illustrates the relative amount of class of protrusions on neurons transfected with the respective construct. Note that constitutively active forms of Ras elicit a similar phenotype as drebrin and that dominant negative Ras partially blocks drebrin-induced filopodial outgrowth.

DISCUSSION

Localized changes in the organization and dynamics of the actin cytoskeleton are likely to underlie the formation, maintenance and plasticity of synaptic connections (Matus, 2000). A variety of evidence, derived in majority from migrating cells, assigns a general role to small GTP-ases as central regulators of actin dynamics and organization. Members from the family of Rho-GTPase, Rho, Rac and cdc42 direct the formation of stress fibers, lamellipodia and filopodia at the leading edge respectively (Hall, 1998), while Ras appears to be implicated in the release of adhesion (Cuevas et al., 2003; Liu et al., 2001).

We show here that drebrin overexpression elicits cell retraction which is accompanied by the collapse of stress fibers and the dissipation of vinculin clusters in fibroblasts, a phenotype reminiscent of Ras transformation (Matsumoto et al., 1997). As could be expected, overexpression of drebrin and constitutively active Ras had synergic effect on cell morphology. In contrast drebrin-induced cell retraction was blocked by co-expression of dominant negative Ras. This inhibiting effect was mediated via the C-terminal part of drebrin which contains a polyproline site and a SH3 domain since cell retraction triggered by deletion mutants lacking this domain could not be blocked by dominant negative Ras co-expression.

In addition, endogenous drebrin is relocated to stress fibers in quiescent fibroblasts stimulated with EGF. Moreover, activation of the Ras-MAPK pathway enhanced drebrin-actin association and drebrin binding to actin is prevented by inhibition of the MAP-kinase pathway as seen in an immunoprecipitation assay.

In summary, these results demonstrate that activation of the Ras-MAP-kinase pathway elicits binding of drebrin to F-actin and subsequent collapse of stabilizing F-actin networks together with the dissipation of vinculin clusters in fibroblasts. Drebrin has been shown to compete with actin-bundling protein for actin binding (Ishikawa et al., 1994) and to inhibit actomyosin interaction (Hayashi et al., 1996). Both these properties would conceivably result in a loss of tension exerted on focal adhesion, which has been shown to disrupt adhesion complex assemblies (BurrIDGE and Chrzanowska-Wodnicka, 1996).

It was especially noticeable that the overexpressed drebrin was to a large part assembled at or at least near the plasma membrane. Drebrin has been previously reported to be a prominent constituent of the actin cytoskeleton at the plasma membrane interface using F-actin blot overlays (Luna et al., 1997). It is therefore conceivable that drebrin may play a substantial role in organizing the actin cytoskeleton of the cell cortex.

In neurons, activation of Ras had profound effect on both dendrite and dendritic spines architecture. Neurons overexpressing constitutively active Ras often exhibited short dendrites, indicating that sustained Ras activation destabilizes the microtubule arrays underlying dendritic shafts.

Activation of the Ras-MAP-kinase pathway by BDNF or spaced stimuli has been shown to induce morphological plasticity in dendritic spines (Wu et al., 2001). In addition, activation of EphB2 receptors, which was shown to downregulate MAP-kinase signaling (Elowe et al., 2001), triggers the premature formation of spine and the stabilization of mature spine structures (Ethell et al., 2001). Here we provide further evidence for a Ras-dependent regulation of dendritic spines stability. Expression of constitutively active Ras in hippocampal neurons triggered growth of filopodia and formation of polymorphic protrusion from dendrites, reminiscent of drebrin-induced destabilization of dendritic spines. Dominant-negative Ras, in contrary, had no noticeable effect on neuron morphology, however, it was able to partially block drebrin-induced formation of polymorphic spines and filopodia, suggesting that in neurons, like in fibroblasts, drebrin binding to actin is regulated by Ras.

Thus it appears that the Ras-MAP-kinase signaling cascade is involved in setting the levels of plasticity in mature dendritic spines.

We therefore propose the following model for Ras dependent regulation of dendritic spine stability; both drebrin and actin cross-linking proteins exist as components of the actin cytoskeleton at the postsynapse. Upon Ras activation, the portion of drebrin bound F-actin rises with the consequence of a collapse of the actin networks and focal adhesions corresponding to a destabilization of dendritic spine structures and to the

formation of polymorphic spines and filopodia. In contrary, downregulation of Ras would raise the ratio of bundled actin within a spine, thus increasing the structural stability of spines (Figure 10).

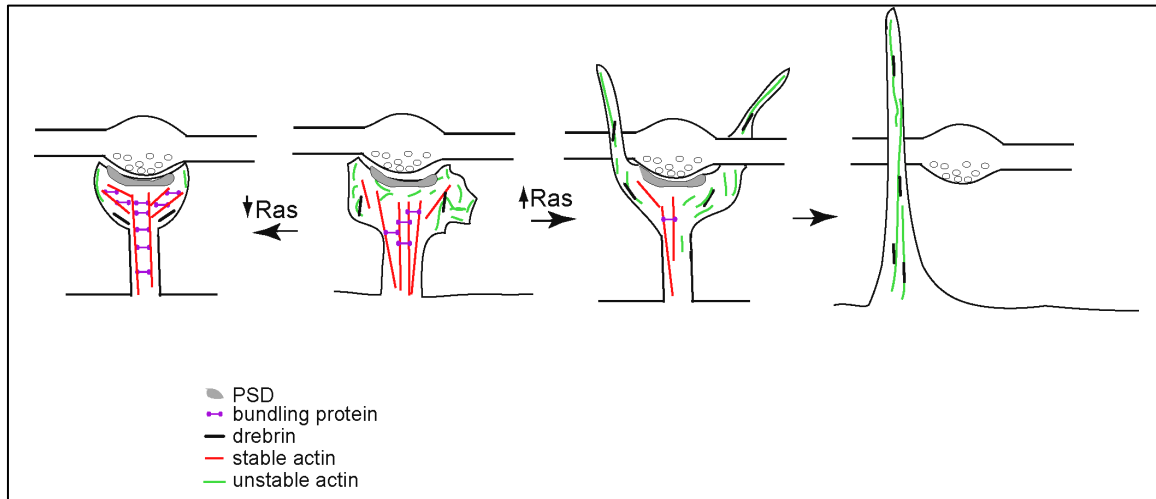


Figure 10: Model for Ras regulated stability of dendritic protrusions:

Stable, cross-linked actin filaments and dynamic, drebrin-bound, actin filaments coexist within dendritic spines. Upon Ras activation, the ratio of drebrin-bound actin increases which concomitantly elicits a destabilization of spine structure leading to the formation of polymorphic spines and filopodia. Conversely when Ras is downregulated, bundled actin predominate, thus stabilizing dendritic spine structure.

CONCLUSIONS AND OUTLOOK

At the outset of this thesis, I outlined the likely primary role that actin binding proteins play in synaptic plasticity.

I identified two major questions:

- 1) How do actin binding proteins influence structure and motility of dendritic spines via the actin cytoskeleton?
- 2) How is synaptic transmission coupled to modifications of synaptic morphologies?

In the first series of experiments I studied the expression of various actin binding proteins in dissociated neurons and especially their localization to dendritic spines. Using this approach I could demonstrate that both, proteins involved in fast actin dynamics, as well as proteins involved in the formation, anchorage and maintenance of stable actin filaments, are fundamental components of the actin cytoskeleton within dendritic spines further arguing that there are two distinct pools of actin within dendritic spines, one characterized by fast actin turnover, evocative of lamellipodial structures at the leading edge of motile cells and a stable one assembled in bundled actin filaments arrays .

In the second set of experiments I examined the potential role of stable actin filaments arrays in determining dendritic spine structure. By using drebrin, a protein involved in regulating the dissociation of cross-linking proteins from actin, as an example, I could show that a pool of stable, cross-linked actin is essential to maintain basic dendritic spine structure. Indeed, drebrin overexpression was accompanied by a general destabilization of dendritic spine structure, which was concomitant with the displacement of α -actinin from dendritic protrusions.

Finally, I examined the role of Ras activation in mediating drebrin induced destabilization of dendritic protrusions. I demonstrated that Ras regulates drebrin-actin association in fibroblasts and provided preliminary evidence suggesting that this may also apply in neuronal cells.

1) The basic configuration of the actin cytoskeleton in dendritic spines is likely to include two distinct structures:

Although actin filaments have been identified as the primordial cytoskeletal component determining dendritic spine's shapes and dynamics, less is known about their organization or about the regulatory mechanisms orchestrating their arrangements in these structures. Actin assembly, structures and dynamics are linked to a myriad of actin binding proteins which functions have been assigned to specific tasks the actin cytoskeleton has to perform (reviewed in Ayscough, 1998).

Insight into the distribution of actin binding proteins is thus a straightforward way to extrapolate the nature of actin arrays or actin networks within a cellular structure and to anticipate what kind of regulatory pathway are most likely to influence them.

In a first set of experiments, I used precisely this approach and examined the distribution of several actin binding proteins in order to make assumptions about the organization of the actin cytoskeleton within dendritic spines.

This initial study revealed that the composition of actin binding proteins in dendritic spines encloses molecules implicated in the regulation of fast actin dynamics in lamellipodia at the leading edge of migrating cells (reviewed in Pollard and Borisy, 2003) and further confirmed the presence of proteins involved in the formation and maintenance of stable actin arrangement (reviewed in Ayscough, 1998)) in those structures. This finding further enforces the notion, that the actin cytoskeleton is composed of two distinct pools within spines, one that is highly motile and one that is highly stable (Allison et al., 2000; Cohen et al., 1985; Fischer et al., 1998; Landis and Reese, 1983).

Considering the identity of some proteins found in spine heads; cofilin, the Arp2/3 complex and gelsolin, it is reasonable to presume that at least a fraction of actin is organized in lamellopodial-like structures (Pollard and Borisy, 2003). These arrays are most likely to underlie the highly motile corona surrounding spine heads (Fischer et al., 1998). The physiological role of these motile lamellipodia surrounding spines is still elusive; however, it is tempting to assume that, in analogy to moving fibroblasts, it might operate as a monitoring device screening extracellular cues within the environment of a respective spine.

In addition, the distribution of major element of actin stabilizing structures and especially the close association of drebrin, which is involved in controlling actin based stable structures (Ishikawa et al., 1994; Sasaki et al., 1996) with spines, anticipates a major role for F-actin bundles in controlling spine morphology.

The deduction of this study implies consequently that the organization and dynamics of actin in dendritic spine structure and surface motility and morphogenesis in non-neuronal cells are governed by the same actin binding proteins and thus conceivably by equivalent regulatory mechanisms.

The challenge, therefore, is to understand how synaptic activity and/or neuromodulators regulate the function of these same actin binding proteins at central nervous system synapses.

2) Stable actin arrays are essential for determining dendritic spines structural integrity:

Having concluded that both motile and stabilized actin structures are present within dendritic spines, I used drebrin as an example to designate the potential role of stable actin arrays in shaping synapses.

This study revealed a fundamental role for bundled F-actin structures in maintaining dendritic spine structural stability since displacement of cross-linking proteins by drebrin is accompanied by disintegration of the basic configuration of dendritic spines, which consists of a bulbous head connected to the dendritic shaft by a narrow neck, and the formation of irregular and highly plastic dendritic protrusions.

In a final study I demonstrated that drebrin-actin association is controlled by the Ras-MAPK pathway in fibroblasts and performed preliminary experiments which suggest that the same regulatory mechanism may also apply to neurons.

In neurons Ras is present in the PSD (Walikonis et al., 2000), and can be activated by both repeated depolarization and brain derived neurotrophic factor (BDNF) (Wu et al., 2001). BDNF has previously been proposed to act as a synaptic morphogen as it is particularly well suited to act as a neuronal activity related molecular cue. Indeed BDNF expression and release are modulated by neuronal activity. BDNF mRNA is strongly increased in rat hippocampus following seizure activity (Zafra et al., 1990) or

LTP-induction (Castren et al., 1993). In addition, blockade of visual inputs, a paradigm accompanied by the reversible loss of dendritic spines (Valverde, 1971), results in a rapid downregulation BDNF in the rat visual cortex and exposing dark-reared mice to light reverses these changes (Castren et al., 1992).

Therefore the BDNF-Ras-MAPK-drebrin pathway might indeed provide a link between synaptic activity and morphological plasticity of the postsynapse.

3) Other molecular mechanisms regulating spine stability:

A small GTPase closely related to Ras, Rap, which also activates the MAPK-pathway has also been implicated in the regulation of spine structure. Overexpression of SPAR, a Rap-GAP, causes the enlargement of spine head and, intriguingly, triggered the formation of irregular, branched spines. In contrary, a mutant of SPAR which can no longer inhibit Rap activity, produced elongation and thinning of spines (Pak et al., 2001). This data suggests that balanced Rap signaling is required for the maintenance of dendritic spine stability.

Overexpression of constitutively active RhoA, the molecular counterpart of Ras, reduces dramatically spine density, however only on a subset of neurons. Inhibition of RhoA by C3-transferase in contrast increased spine density or triggered elongation of spine necks and destabilization of spine structures, again only in a subset of neurons (Tashiro et al., 2000).

It appears that lamellipodial arrays are also of major importance in sustaining spine structures. Expression of dominant negative Rac, a small GTPase involved in regulating lamellipodia formation in moving fibroblasts, induces complete loss of protrusions from dendrites. On the contrary transgenic mice that express constitutively active Rac develop a surplus of dendritic spines of smaller size than usual in Purkinje cells (Luo et al., 1996). Similarly, overexpression of activated Rac in hippocampal neurons produced a net increase in dendritic protrusions including filopodia and membrane ruffles (Nakayama et al., 2000; Tashiro et al., 2000). Moreover, Kalirin-7, a Rac-GEF, increases the size and the density of spines in cortical neurons (Penzes et al., 2001), further indicating that balanced Rac activity and

preservation of lamellipodia on spine heads is important for the maintenance of spine structures.

In line with this idea, mice with a genetic disruption of LIM kinase 1 (LIMK-1), which negatively regulates the severing activity of ADF/cofilin (Arber et al., 1998), which in turns is involved in controlling actin treadmilling in lamellipodia (Pollard and Borisy, 2003), display shortened and enlarged spines (Theriot, 1997).

Taken together these data suggest that multiple pathways control morphogenesis and/or dendritic spine stability. This is not surprising, considering the complexity of spine structure and their dynamic behavior over both short and long timescales.

However, molecular insights into spine formation, maintenance and plasticity should eventually enable us to manipulate dendritic spines in vivo using genetic approaches, in order to address the long-standing questions: What role do spine in experience-dependent adaptation of the brain and why are they plastic?

In this context it will be crucial to identify the primary factors that determine the stability or plasticity of a particular spine.

EXPERIMENTAL PROCEDURE

Generation of constructs:

Extraction of drebrin A and drebrin E1 cDNA's:

Drebrin A and drebrin E1 cDNA were extracted from RNA-samples by RT-PCR. Total RNA was isolated from embryonic brain or adult rat hippocampus using TRizol reagent (LifeTechnologies:Cat.No.15596-026) according to the manufacturers instruction. cDNA's were generated using Superscript reverse transcriptase (SUPERScriptTMII from lifetech Cat.No. 18064-022) and 2 nM of drebrin specific reverse primer .

Drebrin A and drebrin E cDNAs were amplified with specific 5' and 3' primer with NcoI overhangs using proofreading Pfx –DNA polymerase. cDNAs were digested for two hours with NcoI and subcloned into the NcoI site of the p β actin-eGFP vector that drives constitutive expression in hippocampal neurons.

Generation of GFP-tagged drebrin mutants:

Drebrin A cDNA was used for all the deletion mutants shown in Figure 10 in part 2. Briefly, artificial terminal NcoI sites were added to fragments from amino acid 1-233, 1-317, 233-707, 317-707, 233-364 and 233-317 via PCR using proofreading Pfx – DNA polymerase and fragments were subsequently subcloned into the NcoI site of the p β actin-eGFP vector.

Generation of GFP-tagged drebrin mutants:

Annealed primers encoding the HA-coding sequence with a 3' NcoI site were inserted into the HindIII/ RsrII site of the p β actin vector. Deletion mutants were amplified by PCR using proofreading Pfx –DNA polymerase and fragments were subsequently subcloned into the NcoI site of the p β actin- HA vector.

Cell Culture, Transfection, and microscopy:*Dispersed hippocampal neurons:*

Cultures of hippocampal neurons were prepared from E18 rats as described previously according to Banker. Briefly, hippocampi were dissected from E18 rats and seed onto poly-lysine coated coverslips. Cultures were grown on top of a Glia feeder layer with serum free medium for three weeks and more.

*Transfection:**1) DOTAP transfection:*

Dissected, trypsinized and triturated hippocampal neurons from E18 rats were put in suspension in MEM containing 0.6% glucose (1×10^6 cells in 3 ml medium) Cells were added to 3ml Ca^{2+} and Mg^{2+} -free HBSS (Hank's balanced salt solution; GIBCO), buffered with 10mM HEPES pH 7.2, supplemented with 24 μl DOTAP (Roche). Cells were incubated for ten minutes at 37°C before adding $3\mu\text{g}$ DNA. Cells were plated out after 1 hour incubation at 37°C .

2) EFFECTENE (=late) transfection:

For late transfection experiment, plated hippocampal neurons were transfected with EFFECTENE (Qiagen, 301 425). Briefly; per coverslip 100ng DNA were mixed with 30 μl Buffer EC and 0.4 μl enhancer and incubated at RT for 5 min before adding 0.5 μl effectene. After further 5 min incubation at RT, DNA-effectene complex was added to 200 μl previously collected conditioned medium +100 μM APV. Coverslips were flipped onto parafilm and immediately covered with transfection mix. Coverslips were incubated for further 90 min in an incubator before being flipped back to the GLIA feeder layer.

REF-52 cells:

Rat embryonic fibroblasts (REF-52) were maintained in DMEM with 10% fetal calf serum. REF -52 cells were transfected with Fugene-6 according to the manufacturers instructions.

Microscopy:

For microscopy the coverslips were mounted on custom-made observation chambers (Type1, life imaging services, Olten, Switzerland) and imaged at 37°C in Tyrodes solution pH 7.3 on a Leica DM-IRBE inverted microscope using high aperture oil-immersion lenses and GFP, YFP and CFP-optimized filter set (Chroma Technologies, Brattleboro, Vermont) and a Micromax cooled CCD camera (Princeton Instriments, treston, New Jersey). Confocal microscopy was performed using a real time spinning disk-based confocal microscope (Yokogawa Instruments, Tokyo, Japan). Pictures were captured using MetaMorph imaging Software (Universal Imaging Corporation, West Chester, PA).

Image analysis and quantifications:

In a blind analysis globular, polymorphic spines and filopodia were counted on 50 μ m portions of dendrites. After counting, the code was broken and percentage of protrusion classes per constructs was calculated using Microsoft Excel. Spines outlines were generated from threshold images using an edge-detection function of MetaMorph Software. Changes in shapes as shown in Figure 5 were assessed using the 'shape factor' algorithm of MetaMorph Software as described (Fischer et al., 2000).

Fixation and staining of neurons:

For immunocytochemistry cells were fixed for 10 minutes at 37°C with 4% paraformaldehyde and 4% sucrose in PBS (phosphate buffer saline), permeabilized with 0.25% Triton X-100 in PBS for 10 minutes at room temperature and blocked with 5% NGS (normal goat serum) and 1% BSA in PBS for 1 hour. After immunodetection cells were mounted in Mowiol (Hoechst).

Immunoprecipitation:

Fibroblasts were grown 10cm Ø culture dish until confluent. Cells were washed 3x with PBS (+Ca²⁺) before being serum starved for 20 hour. After treatments cells were washed carefully with 1ml cold PBS before 300µl NP-40 extraction Buffer was added. Cells were incubated in NP-40 extraction Buffer for 5 min on ice before being scraped with a police-man. Insoluble fractions were removed by centrifugation at 1700g for 15 min in cold room. Between 0.5 and 1.0 mg of total proteins from cell extract were incubated with 1-2 µg of antibody in 500-1000µl NP-40 extraction Buffer and rotate at 4°C for 1 hour before 25µl of protein-A-sepharose were added. Samples were rotated at 4°C for an additional hour. Protein-beads were washed 3x with 1 ml cold extraction buffer and once with 1ml TNE. 30 µl of 2x SDS loading buffer were added and mixed by vortexing. Samples were heat at 95°C for 10 minutes and vortex once during this time to remove immonoprecipitated proteins from beads. Samples were spin down and the supernatant loaded on 8-10% SDS-PAGE gel.

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Date of Birth: 13.07.1972
Nationality: French/Swiss
Marital Status: single

Education/Research experience:

- 1998-present: Dissertation (Ph.D.) in the lab of Prof. Andrew Matus
Department of Neurobiology,
Friedrich Miescher Institute, Basel, Switzerland
“Cytoskeletal and morphological determinants of dendritic spines”
- 1997: Master of Science in biology:
 - Cellular Biology
 - Microbiology
 - Biophysical Chemistry
 - Biochemistry
- 1996-1997: Diploma thesis in the Lab of Prof. John G. Nicholls
Department of Pharmacology
Biozentrum, University of Basel
“Development and regeneration of monoaminergic neurons in the central nervous system of the newborn opossum (monodelphis domestica)”
- 1992-1995: Biology course at the “Biozentrum”,
University of Basel, Switzerland
- 1991: Matura at the “Mathematisches-Naturwissenschaftliches-Gymnasium”, Basel, Switzerland

Skills:

- **Molecular biology:** Site directed mutagenesis, RT-PCR, Southern, Northern and Western blot analysis, In Situ hybridization
- **Biochemistry:** immuno-precipitation and subcellular fractionation
- **Histology:** including Golgi impregnation
- **Cell culture:** including primary hippocampal cultures, organotypic slice cultures, whole neonatal CNS explants, transfection of primary cultures and organotypic slice culture.
- **Microscopy:** confocal microscopy and live cell imaging
- **Animal work:** mouse handling and mouse behavior tests including open-field, elevated Plus-Maze, T-maze and object recognition test.
- **IT:** MS Office software, Adobe Photoshop and Illustrator, Metamorph,
- **Languages:** French, German, English

Grants:

- Roche Research foundation: 08.1998- 08.1999
Stiftung der Basler Chemischen Industrie: 08.1999- 08.2001

Courses and Meetings:

- EMBL/FENS courses: Mouse Transgenics and Behaviour
Centre for neuroscience,
University of Edinburgh
16-29th August 1999, Edingburgh (UK)
- FENS-Meeting: Federation of European Neuroscience Societies 2000
meeting, Brighton (UK)
- Neurex meeting: Réunion Neurologique joint 2000 meeting, Freiburg (D)
- EMBO courses: Organizing the brain: Genes, neurons and circuits
February 5-8th, 2002, Ascona, Switzerland

Publications:

Three-Dimensional Visualization of the distribution, Growth, and Regeneration of monoaminergic Neurons in Whole Mounts of immature Mammalian CNS

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The Journal of Comparative Neurology 390: 427-438 (1998)

Imaging the actin cytoskeleton in living cells, in molecular cloning, A laboratory manual.

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Drebrin regulates the basic structure of dendritic spines

Virginie Biou and Andrew Matus

Submitted

Effects of V22M- Ras and V12 K-ras4B on actin cytoskeleton in neuronal and non-neuronal cells- a comparison study.

Arkadiusz Welman, Virginie Biou and Andrew Matus

In preparation

The actin-binding protein drebrin regulates the morphological plasticity of dendritic spines via a Ras-dependent pathway.

Virginie Biou and Andrew Matus

In preparation